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| (54) Title: A CELLULASE PREPARATION COMPRISING AN ENDOGLUCANASE ENZYME (57) Abstract A cellulase preparation consisting essentially of a homogeneous endoglucanase component which is immunoreactive with an antibody raised against a highly purified ~43kD endoglucanase derived from <i>Humicola insolens</i> , DSM 1800, or which is homogeneous to said ~43kD endoglucanase, may be employed in the treatment cellulose-containing fabrics for harshness reduction or colour clarification or to provide a localized variation in the colour of such fabrics, or it may be employed in the treatment of paper pulp. | | |

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A CELLULASE PREPARATION COMPRISING AN ENDOGLUCANASE ENZYME

FIELD OF INVENTION

The present invention concerns a cellulase preparation comprising a single-component endoglucanase, a detergent additive comprising the cellulase preparation, a detergent composition containing the cellulase preparation as well as methods of treating cellulose-containing fabrics with the cellulase preparation.

BACKGROUND OF THE INVENTION

10 It is well known in the art that repeated washing of cotton-containing fabrics generally causes a pronounced, unpleasant harshness in the fabric, and several methods for overcoming this problem have previously been suggested in the art. For example GB 1,368,599 of Unilever Ltd. teaches the use
15 of cellulytic enzymes for reducing the harshness of cotton-containing fabrics. Also, US 4,435,307 (of Novo Industri A/S) teaches the use of a cellulytic enzyme derived from Humicola insolens as well as a fraction thereof, designated AC_xI, as a harshness reducing detergent additive. Other uses of cellulytic
20 enzymes mentioned in the art involve soil removal from and colour clarification of fabric (cf. for instance EP 220 016), providing increasing water absorption (JP-B-52-48236) and providing a localized variation in colour to give the treated fabrics a "stone-washed" appearance (EP 307,564). Cellulytic
25 enzymes may furthermore be used in the brewing industry for the degradation of β -glucans, in the baking industry for improving the properties of flour, in paper pulp processing for removing the non-crystalline parts of cellulose, thus increasing the proportion of crystalline cellulose in the pulp, and for
30 improving the drainage properties of pulp, and in animal feed for improving the digestibility of glucans.

The practical exploitation of cellulytic enzymes has, to some extent, been set back by the nature of the known cellulase

preparations which are often complex mixtures. It is difficult to optimis the production of multiple enzyme systems and thus to implement industrial cost-effective production of cellulytic enzymes, and their actual use has been hampered by difficulties
5 arising from the need to apply rather large quantities of the cellulytic enzymes to achieve the desired effect on cellulosic fabrics.

The drawbacks of previously suggested cellulase preparations may be remedied by using preparations comprising
10 a higher amount of endoglucanases. A cellulase preparation enriched in endoglucanase activity is disclosed in WO 89/00069.

SUMMARY OF THE INVENTION

A single endoglucanase component has now been isolated which exhibits favourable activity levels relative to
15 cellulose-containing materials.

Accordingly, the present invention relates to a cellulase preparation consisting essentially of a homogenous endoglucanase component which is immunoreactive with an antibody raised against a highly purified ~43 kD endoglucanase
20 derived from Humicola insolens, DSM 1800, or which is homologous to said ~43 kD endoglucanase.

The finding that this particular endoglucanase component of cellulase is advantageous for the treatment of cellulose-containing materials is of considerable practical significance:
25 it permits a cost-effective production of the cellulase, e.g. by employing recombinant DNA techniques for producing the active component, and makes the actual effective application of the enzyme feasible in that a smaller quantity of the cellulase preparation is requested to produce the desired effect on
30 cellulosic materials.

DETAILED DISCLOSURE OF THE INVENTION

The cellulase preparation of the invention is advantageously one in which the endoglucanase component exhibits a

CMC-endoase activity of at least about 50 CMC-endoase units per mg of total protein.

In the present context, the term "CMC-endoase activity" refers to the endoglucanase activity of the endoglucanase component in terms of its ability to degrade cellulose to glucose, cellobiose and triose, as determined by a viscosity decrease of a solution of carboxymethyl cellulose (CMC) after incubation with the cellulase preparation of the invention, as described in detail below.

10 Preferred cellulase preparations of the invention are those in which the endoglucanase component exhibits a CMC-endoase activity of at least about 60, in particular at least about 90, CMC-endoase units per mg of total protein. In particular, a preferred endoglucanase component exhibits a CMC-15 endoase activity of at least 100 CMC-endoase units per mg of total protein.

The CMC-endoase (endoglucanase) activity can be determined from the viscosity decrease of CMC, as follows:

A substrate solution is prepared, containing 35 g/l CMC 20 (Hercules 7 LFD) in 0.1 M tris buffer at pH 9.0. The enzyme sample to be analyzed is dissolved in the same buffer.

10 ml substrate solution and 0.5 ml enzyme solution are mixed and transferred to a viscosimeter (e.g. Haake VT 181, NV sensor, 181 rpm), thermostated at 40°C.

25 Viscosity readings are taken as soon as possible after mixing and again 30 minutes later. The amount of enzyme that reduces the viscosity to one half under these conditions is defined as 1 unit of CMC-endoase activity.

SDS polyacrylamide gel electrophoresis (SDS-PAGE) and 30 isoelectric focusing with marker proteins in a manner known to persons skilled in the art were used to determine the molecular weight and isoelectric point (pI), respectively, of the endoglucanase component in the cellulase preparation of the invention. In this way, the molecular weight of a specific 35 endoglucanase component was determined to be \approx 43 kD. The isoelectric point of this endoglucanase was determined to be about 5.1. The immunochemical characterization of the

endoglucanase was carried out substantially as described in WO 89/00069, establishing that the end glucanase is immunoreactive with an antibody raised against highly purified ~43 kD endoglucanase from Humicola insolens, DSM 1800. The cellobio-
5 hydrolase activity may be defined as the activity towards cellobiose p-nitrophenyl. The activity is determined as μ mole nitrophenyl released per minute at 37°C and pH 7.0. The present endoglucanase component was found to have essentially no cellobiohydrolase activity.

10 The endoglucanase component in the cellulase preparation of the invention has initially been isolated by extensive purification procedures, i.a. involving reverse phase HPLC purification of a crude H. insolens cellulase mixture according to US 4,435,307 (cf. Example 1 below). This procedure has
15 surprisingly resulted in the isolation of a ~43 kD endoglucanase as a single component with unexpectedly favourable properties due to a surprisingly high endoglucanase activity.

In another aspect, the present invention relates to an
20 enzyme exhibiting endoglucanase activity (in the following referred to as an "endoglucanase enzyme"), which enzyme has the amino acid sequence shown in the appended Sequence Listing ID#2, or a homologue thereof exhibiting endoglucanase activity. In the present context, the term "homologue" is intended to
25 indicate a polypeptide encoded by DNA which hybridizes to the same probe as the DNA coding for the endoglucanase enzyme with this amino acid sequence under certain specified conditions (such as presoaking in 5xSSC and prehybridizing for 1 h at ~40°C in a solution of 20% formamide, 5xDenhardt's solution, 50
30 mM sodium phosphate, pH 6.8, and 50 μ g of denatured sonicated calf thymus DNA, followed by hybridization in the same solution supplemented with 100 μ M ATP for 18 h at ~40°C). The term is intended to include derivatives of the aforementioned sequence obtained by addition of one or more amino acid residues to
35 either or both the C- and N-terminal of the native sequence, substitution of one or more amino acid residues at one or more sites in the native sequence, deletion of one or more amino

acid residues at either or both ends of the native amino acid sequence or at one or more sites within the native sequence, or insertion of one or more amino acid residues at one or more sites in the native sequence.

5 The endoglucanase enzyme of the invention may be one producible by species of Humicola such as Humicola insolens e.g. strain DSM 1800, deposited on 1 October 1981 at the Deutsche Sammlung von Mikroorganismen, Mascheroder Weg 1B, D-3300 Braunschweig, FRG, in accordance with the provisions of the Budapest
10 Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (the Budapest Treaty).

In a further aspect, the present invention relates to an endoglucanase enzyme which has the amino acid sequence shown in
15 the appended Sequence Listing ID#4, or a homologue thereof (as defined above) exhibiting endoglucanase activity. Said endoglucanase enzyme may be one producible by a species of Fusarium, such as Fusarium oxysporum, e.g. strain DSM 2672, deposited on 6 June 1983 at the Deutsche Sammlung von
20 Mikroorganismen, Mascheroder Weg 1B, D-3300 Braunschweig, FRG, in accordance with the provisions of the Budapest Treaty.

Furthermore, it is contemplated that homologous endoglucanases may be derived from other microorganisms producing cellulolytic enzymes, e.g. species of Trichoderma,
25 Myceliophthora, Phanerochaete, Schizophyllum, Penicillium, Aspergillus, and Geotricum.

The present invention also relates to a DNA construct comprising a DNA sequence encoding an endoglucanase enzyme as described above, or a precursor form of the enzyme. In
30 particular, the DNA construct has a DNA sequence as shown in the appended Sequence Listings ID#1 or ID#3, or a modification thereof. Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the endoglucanase, but which correspond
35 to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and therefore,

possibly, a different protein structure which might give rise to an endoglucanase mutant with different properties than the native enzyme. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, 5 addition of one or more nucleotides at either end of the sequence, or deletion of one or more nucleotides at either end or within the sequence.

The DNA construct of the invention encoding the endoglucanase enzyme may be prepared synthetically by 10 established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, 15 e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

A DNA construct encoding the endoglucanase enzyme or a precursor thereof may, for instance, be isolated by establishing a cDNA or genomic library of a cellulase-producing 20 microorganism, such as Humicola insolens, DSM 1800, and screening for positive clones by conventional procedures such as by hybridization using oligonucleotide probes synthesized on the basis of the full or partial amino acid sequence of the endoglucanase in accordance with standard techniques (cf. 25 Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd. Ed., Cold Spring Harbor, 1989), or by selecting for clones expressing the appropriate enzyme activity (i.e. CMC-endoase activity as defined above), or by selecting for clones producing a protein which is reactive with an antibody against 30 a native cellulase (endoglucanase).

Finally, the DNA construct may be of mixed synthetic and genomic, mixed synthetic and cDNA or mixed genomic and cDNA origin prepared by ligating fragments of synthetic, genomic or cDNA origin (as appropriate), the fragments corresponding to 35 various parts of the entire DNA construct, in accordance with standard techniques. The DNA construct may also be prepared by polymerase chain reaction using specific primers, for instance

as described in US 4,683,202 or R.K. Saiki et al., Science 239, 1988, pp. 487-491.

The invention further relates to a recombinant expression vector into which the DNA construct of the invention is inserted. This may be any vector which may conveniently be subjected to recombinant DNA procedures, and the choice of vector will often depend on the host cell into which it is to be introduced. Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the endoglucanase should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the endoglucanase, the promoter and the terminator, respectively, and to insert them into suitable vectors are well known to persons skilled in the art (cf., for instance, Sambrook et al., op.cit.).

The invention also relates to a host cell which is transformed with the DNA construct or the expression vector of the invention. The host cell may for instance belong to a species of Aspergillus, most preferably Aspergillus oryzae or Aspergillus niger. Fungal cells may be transformed by a process involving protoplast formation and transformation of the protoplasts followed by regeneration of the cell wall in a manner known per se. The use of Aspergillus as a host microorganism is described in EP 238,023 (of Novo Industri A/S), the contents of which are hereby incorporated by ref-

erence. The host cell may also be a yeast cell, e.g. a strain of Saccharomyces cerevisiae.

Alternatively, the host organism may be a bacterium, in particular strains of Streptomyces and Bacillus, and E. coli.

5 The transformation of bacterial cells may be performed according to conventional methods, e.g. as described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989.

The present invention further relates to a process for
10 producing an endoglucanase enzyme of the invention, the process comprising culturing a host cell as described above in a suitable culture medium under conditions permitting the expression of the endoglucanase enzyme, and recovering the endoglucanase enzyme from the culture. The medium used to
15 culture the transformed host cells may be any conventional medium suitable for growing the host cells in question. The expressed endoglucanase may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by
20 centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

By employing recombinant DNA techniques as indicated
25 above, techniques of protein purification, techniques of fermentation and mutation or other techniques which are well known in the art, it is possible to provide endoglucanases of a high purity.

The cellulase preparation or endoglucanase enzyme of the
30 invention may conveniently be added to cellulose-containing fabrics together with other detergent materials during soaking, washing or rinsing operations. Accordingly, in another aspect, the invention relates to a detergent additive comprising the cellulase preparation or endoglucanase enzyme of the invention.

35 The detergent additive may suitably be in the form of a non-dusting granulate, stabilized liquid or protected enzyme. Non-dusting granulates may be produced e.g. according to US

4,106,991 and 4,661,452 (both to Novo Industri A/S) and may optionally be coated by methods known in the art. Liquid enzyme preparations may, for instance, be stabilized by adding a polyol such as propylene glycol, a sugar or sugar alcohol, 5 lactic acid or boric acid according to established methods. Other enzyme stabilizers are well known in the art. Protected enzymes may be prepared according to the method disclosed in EP 238,216.

The detergent additive may suitably contain 1 - 500, 10 preferably 5 - 250, most preferably 10 - 100 mg of enzyme protein per gram of the additive. It will be understood that the detergent additive may further include one or more other enzymes, such as a protease, lipase, peroxidase or amylase, conventionally included in detergent additives.

15 According to the invention, it has been found that when the protease is one which has a higher degree of specificity than Bacillus lentus serine protease, an increased storage stability of the endoglucanase enzyme is obtained. (For the present purpose, a protease with a higher degree of specificity 20 than B. lentus serine protease is one which degrades human insulin to fewer components than does the B. lentus serine protease under the following conditions: 0.5 ml of a 1 mg/ml solution of human insulin in B and R buffer, pH 9.5, is incubated with 75 μ l enzyme solution of 0.6 CPU [cf. Novo 25 Nordisk Analysis Methods No. AF 228/1] per litre for 120 min. at 37°C, and the reaction is quenched with 50 μ l 1N HCl). Examples of such proteases are subtilisin Novo or a variant thereof (e.g. a variant described in US 4,914,031), a protease derivable from Nocardia dassonvillei NRRL 18133 (described in 30 WO 88/03947), a serine protease specific for glutamic and aspartic acid, producible by Bacillus licheniformis (this protease is described in detail in co-pending International patent application No. PCT/DK91/00067), or a trypsin-like protease producible by Fusarium sp. DSM 2672 (this protease is 35 described in detail in WO 89/06270).

In a still further aspect, the invention relates to a detergent composition comprising the cellulase preparation or endoglucanase enzyme of the invention.

Detergent compositions of the invention additionally
5 comprise surfactants which may be of the anionic, non-ionic, cationic, amphoteric, or zwitterionic type as well as mixtures of these surfactant classes. Typical examples of anionic surfactants are linear alkyl benzene sulfonates (LAS), alpha olefin sulfonates (AOS), alcohol ethoxy sulfates (AES) and
10 alkali metal salts of natural fatty acids. It has, however, been observed that the endoglucanase is less stable in the presence of anionic detergents and that, on the other hand, it is more stable in the presence of non-ionic detergents or certain polymeric compounds such as polyvinylpyrrolidone,
15 polyethylene glycol or polyvinyl alcohol. Consequently, the detergent composition may contain a low concentration of anionic detergent and/or a certain amount of non-ionic detergent or stabilising polymer as indicated above.

Detergent compositions of the invention may contain
20 other detergent ingredients known in the art as e.g. builders, bleaching agents, bleach activators, anti-corrosion agents, sequestering agents, anti soil-redeposition agents, perfumes, enzyme stabilizers, etc.

The detergent composition of the invention may be
25 formulated in any convenient form, e.g. as a powder or liquid. The enzyme may be stabilized in a liquid detergent by inclusion of enzyme stabilizers as indicated above. Usually, the pH of a solution of the detergent composition of the invention will be 7-12 and in some instances 7.0-10.5. Other detergent enzymes
30 such as proteases, lipases or amylases may be included the detergent compositions of the invention, either separately or in a combined additive as described above.

The softening, soil removal and colour clarification effects obtainable by means of the cellulase preparation of the
35 invention generally require a concentration of the cellulase preparation in the washing solution of 0.0001 - 100, preferably 0.0005 - 60, and most preferably 0.01 - 20 mg of enzyme protein

per liter. The detergent composition of the invention is typically employed in concentrations of 0.5 - 20 g/l in the washing solution. In general, it is most convenient to add the detergent additive in amounts of 0.1 - 5% w/w or, preferably, 5 in amounts of 0.2 - 2% of the detergent composition.

In a still further aspect, the present invention relates to a method of reducing the rate at which cellulose-containing fabrics become harsh or of reducing the harshness of cellulose-containing fabrics, the method comprising treating cellulose-
10 containing fabrics with a cellulase preparation or endoglucanase enzyme as described above. The present invention further relates to a method providing colour clarification of coloured cellulose-containing fabrics, the method comprising treating coloured cellulose-containing fabrics with a cellulase
15 preparation or endoglucanase, and a method of providing a localized variation in colour of coloured cellulose-containing fabrics, the method comprising treating coloured cellulose-containing fabrics with a cellulase preparation or endoglucanase of the invention. The methods of the invention
20 may be carried out by treating cellulose-containing fabrics during washing. However, if desired, treatment of the fabrics may also be carried out during soaking or rinsing or simply by adding the cellulase preparation or the endoglucanase enzyme to water in which the fabrics are or will be immersed.

25 According to the invention, it has been found that the drainage properties of paper pulp may be significantly improved by treatment with the endoglucanase of the invention without any significant concurrent loss of strength. Consequently, the present invention further relates to a method of improving the
30 drainage properties of pulp, the method comprising treating paper pulp with a cellulase preparation or an endoglucanase enzyme according to the invention. Examples of pulps which may be treated by this method are waste paper pulp, recycled cardboard pulp, kraft pulp, sulphite pulp, or thermomechanical
35 pulp and other high-yield pulps.

The present invention is described in further detail with reference to currently preferred embodiments in the fol-

lowing examples which are not intended to limit the scope of the invention in any way.

EXAMPLES

Example 1

5 Isolation of a ~43 kD endoglucanase from *Humicola insolens*

1. Preparation of a rabbit antibody reactive with a ~43 kD endoglucanase purified from *Humicola insolens* cellulase mixture

Cellulase was produced by cultivating *Humicola insolens* DSM 1800, as described in US 4,435,307, Example 6. The crude
10 cellulase was recovered from the culture broth by filtration on diatomaceous earth, ultrafiltration and freeze-drying of the retentate, cf. Examples 1 and 6 of US 4,435,307.

The crude cellulase was purified as described in WO 89/09259, resulting in the fraction F1P1C2 which was used for
15 the immunization of mice. The immunization was carried out 5 times at bi-weekly intervals, each time using 25 µg protein including Freund's Adjuvant.

Hybridoma cell lines were established as described in Ed Harlow and David Lane, Antibodies. A Laboratory Manual, Cold
20 Spring Harbor Laboratory 1988. The procedure may briefly be described as follows:

After bleeding the mouse and showing that the mouse serum reacts with proteins present in the F1P1C2 fraction, the spleen was removed and homogenized and then mixed with PEG and
25 Fox-river myeloma cells from Hyclone, Utah, USA.

The hybridomas were selected according to the established HAT screening procedure.

The recloned hybridoma cell lines were stabilized. The antibodies produced by these cell lines were screened and
30 selected for belonging to the IgG1 subclass using a commercial mouse monoclonal typing kit from Serotec, Oxford, England. Positive antibodies were then screened for reactivity with F1P1C2 in a conventional ELISA, resulting in the selection of

F4, F15 and F41 as they were all very good in ELISA response but were found to have different response in immunoblotting using crude H. insolens, DSM 1800, cellulase in SDS-PAGE followed by Western Blot, indicating that they recognized 5 different epitopes.

The three antibodies were produced in large quantities in the ascites fluid of CRBF mice. The mouse gammaglobulin was purified from ascites fluid by protein A purification using protein A coupled to Sepharose (Kem.En.Tek., Copenhagen, 10 Denmark).

The different monoclonal gammaglobulins were tested for response in a sandwich ELISA using each monoclonal antibody as the catching antibody, various HPLC fractions of Celluzyme as the antigen, and a rabbit antibody raised against endoglucanase 15 B from Celluzyme as the detection antibody.

To visualize binding in the ELISA, a porcine antibody against rabbit IgG covalently coupled to peroxidase from Dakopatts (Copenhagen, Denmark) and was visualized with OPD(1,2-phenylenediamine, dihydrochloride)/H₂O₂.

20 The highest ELISA response was obtained with the monoclonal antibody F41 which was therefore used in the immunoaffinity purification steps.

The purified mouse gammaglobulin F41 was coupled to 43 g of CNBr-activated Sepharose 4B as described by the manufacturer (Pharmacia, Sweden) followed by washing. 25

2. Immunoaffinity purification of ~43 kD endoglucanase from a H. insolens cellulase mixture

H. insolens cellulase mixture (as described above) was diluted to 3% dry matter, and the pH was adjusted to 3.5 in 15 30 min. at 4 °C. The precipitate was removed by filtration after adjusting the pH to 7.5. Then sodium sulphate was added to precipitate the active enzyme and this was done at 40°C (260 gram per kg at pH 5.5). The precipitate was solubilized with water and filtered. The acid treatment was repeated. Finally, 35 the product was filtered and concentrated by ultrafiltration using a polyvinylsulphonate membrane with a 10.000 Mw cut-off.

The cellulase product was then diluted to 3% dry matter, adjusting the pH to 9.0, and subjected to anion exchange chromatography on a DEAE-Sepharose column as recommended by the manufacturer (Pharmacia, Sweden).

5 The protease-free cellulase product was applied on the F 41 gammaglobulin-coupled Sepharose column described above at pH 8.0 in sodium phosphate buffer.

After application the column was washed with the same buffer containing 0.5 M sodium chloride. The column was then
10 washed with 0.1 M sodium acetate buffer containing 0.5 M sodium chloride, pH 4.5, after which the column was washed in 5 mM sodium acetate buffer, pH 4.5. Finally, the ~43 kD endoglucanase was eluted with 0.1 M citric acid.

Total yield: 25 mg with an endoglucanase activity of
15 1563 CMC-endoase units.

The eluted protein migrates as a single band in SDS-PAGE with an apparent MW of ~43 kD and a pI after isoelectric focusing of about 5.0 to 5.2.

Inactive protein was removed by reverse phase purification.
20

Inactive and active protein was separated by HPLC using a gradient of 2-propanol. Inactive protein elutes at about 25% 2-propanol and the active ~43 kD endoglucanase elutes at 30% 2-propanol, the active endoglucanase being detectable by a CMC-
25 Congo Red clearing zone.

In this way, a total of 0.78 mg active protein was recovered with 122 CMC endoase units. This procedure was repeated 30 times.

The ~43 kD endoglucanase was recovered by first freeze-
30 drying to remove the TFA and propanol and then solubilizing in phosphate buffer.

The endoglucanase activity of the purified material was 156 CMC-endoase units per mg protein and the total yield including freeze-drying was 65% of the endoglucanase activity.

35 The thus obtained ~43 kD enzyme was used to immunise rabbits according to the procedure described by N. Axelsen et al. in A Manual of Quantitative Immunoelectrophoresis,

Blackwell Scientific Publications, 1973, Chapter 23. Purified immunoglobulins were recovered from the antisera by ammonium sulphate precipitation followed by dialysis and ion exchange chromatography on DEAE-Sephadex in a manner known per se.

5 Binding of purified immunoglobulin to the endoglucanase was determined, and the rabbit immunoglobulin AS 169 was selected for further studies.

2. Characterization of the ~43 kD endoglucanase:

Amino acid composition: Using total hydrolysis, the
10 following composition was obtained after amino acid analysis:

| | |
|--------|----|
| Asp | 17 |
| Asn | 15 |
| Thr | 25 |
| Ser | 29 |
| 15 Glu | 6 |
| Gln | 13 |
| Pro | 21 |
| Gly | 32 |
| Ala | 23 |
| 20 Cys | 20 |
| Val | 14 |
| Met | 1 |
| Ile | 7 |
| Leu | 8 |
| 25 Tyr | 6 |
| Phe | 15 |
| Lys | 9 |
| His | 2 |
| Trp | 9 |
| 30 Arg | 12 |

The Mw of the non-glycosylated protein was estimated to be 30,069 based on the amino acid composition. The glycosylation was measured to

| | |
|-----------|----|
| Galactose | 10 |
| 5 Mannose | 28 |

corresponding to a Mw of 6,840, resulting in a total Mw of the endoglucanase of 36,900 (+/- 2,400). The extinction coefficient per mole was estimated as follows:

| | | |
|----|------------|-----------------|
| | Tryptophan | 9 times 5690 |
| 10 | Tyrosine | 6 times 1280 |
| | Cysteins | 20 times 120 |
| | total | 61290 per mole. |

Extinction coefficients are 1.66 at 280 nm corresponding to 1 mg protein per ml. (Reference: S.C.Gill and P. Hippel, Anal. Biochemistry 182, 312-326 (1989).)

The amino acid sequence was determined on an Applied Biosystems 475A Protein Sequenator using Edman degradation. Only one sequence indicated the purity of the protein. The amino acid sequence is shown in the appended Sequence Listing ID#2.

Enzyme properties:

The enzyme is stable between pH 3 and 9.5.

The enzyme does not degrade highly crystalline cellulose or the substrate cellobiose β -p-nitrophenyl, (Cellobiohydrolase substrate), but degrades amorphous cellulose mainly to cellobiose, cellotriose and cellotetraose, indicating that the enzyme may be used to produce cellodextrins from insoluble amorphous cellulose.

The enzyme is active between pH 6.0 and 10.0 with a maximum activity at about 50°C.

Example 2Cloning and expression of the ~43 kD endoglucanase in *Aspergillus oryzae*Partial cDNA:

5 A cDNA library was made from *Humicola insolens* strain DSM 1800 mRNA (Kaplan et al. (1979) Biochem.J. 183, 181-184) according to the method of Okayama and Berg (1982) Mol. Cell. Biol. 2, 161-170. This library was screened by hybridization with radioactively labelled oligonucleotides to filters with
10 immobilized DNA from the recombinants (Gergen et al. (1979) Nucleic Acids Res. 7, 2115-2136). The oligonucleotide probes were made on the basis of amino acid sequences of tryptic fragments of the purified ~43 kD endoglucanase. A colony was found to hybridize to three different probes (NOR 1251, 2048,
15 and 2050) and was isolated. The sequence showed that the inserted 680 bp cDNA coded for the C-terminal 181 aminoacids of the ~43 kD protein and the 3' nontranslated mRNA. A 237 bp long Pvu I -Xho I fragment from this clone was used to probe a Northern blot (as described in Sambrook et al, op. cit., p.
20 7.40-7.42 and p. 7.46-7.48.) with *H. insolens* mRNA and it was shown that the entire ~43 kD mRNA has a length of app. 1100 bp. The same 237 bp fragment was used to probe a genomic library from the same strain.

Genomic clone:

25 A *Humicola insolens* strain DSM 1800 genomic library was made from total DNA prepared by the method of Yelton (M. M. Yelton et al. (1984) Proc. Natl. Acad. Sci. USA. 81. 1470-1474) and partially digested with Sau 3A. Fragments larger than 4 kb were isolated from an agarose gel and ligated to pBR 322
30 digested with Bam HI and dephosphorylated. The ligation products was transformed into *E. coli* MC1000 (Casadaban and Cohen (1980). J. Mol. Biol., 138, 179-207) made r^m by conventional methods. 40.000 recombinants were screened with the 237 bp Pvu I -Xho I partial cDNA fragment described in the

paragraph "partial cDNA". 2 colonies that contained the entire ~43 kD endoglucanase sequence were selected and the gene was sequenced by the dideoxy method using the Sequenase® kit (United States Biochemical Corporation) according to the manufacturer's 5 instructions. The sequence was identical to the sequence of the full length cDNA gene (see the paragraph "full length cDNA" below) except for one intron in the genomic gene.

The genomic gene was amplified by the PCR method using a Perkin-Elmer/Cetus DNA Amplification System according to the 10 manufacturer's instructions. In the 5' end of the gene the primer NOR 2378 was used. This primer is a 25 mer matching the 5' untranslated end of the gene except for one C to T replacement generating a Bcl I site. In the 3' end of the gene the primer NOR 2389 was used. This primer is a 26 mer of which 15 21 bases match the 3' untranslated part of the gene and the 5 bases in the 5' end of the primer completes a Sal I site.

The Aspergillus expression vector pToC 68 was constructed from plasmid p775 (the construction of which is described in EP 238 023) by insertion of the following linkers

20 KFN 514: 5'-AGCTGCGGCCGCGAGGCCGCGGAGGCCA-3'

KFN 515: 3'-CGCCGGCGTCCGCGCGCTCCGGTTCCA-5'

SacII

HindIII

EcoRI NotI StII

KFN 516: 5'-AATTCGCGGCCGCGGCCATGGAGGCC-3'

25 KFN 519: 3'-GCGCCGGCGCCGCTACCTCCGGTTAA-5'

NcoI

The construction of pToC is shown in the appended Fig. 1.

The PCR fragment obtained above was digested with Bcl I and Sal I and inserted into pToC 68 digested with Bam HI and 30 Xho I. The insert of the resulting plasmid (pCaHj 109) was sequenced and shown to be identical to the original clone.

Full length cDNA:

First strand cDNA was synthesized from a specific primer within the known sequence (NOR 2153), and second strand synthesis was made by the method described by Gubler and Hoffman (1983) GENE 25, 263-269. The sequence of the genomic gene made it possible to design a PCR primer to catch the 5' part of the mRNA and at the same time introduce a Bam HI site right in front of the ATG start codon (NOR 2334). By using this primer at the 5' end and NOR 2153 again at the 3' end PCR was performed on the double stranded cDNA product. The full length coding part of the PCR-cDNA was then constructed by cloning the 5' Bam HI - Pvu I fragment from the PCR reaction together with the 3' Pvu I - Eco O109, filled out with Klenow polymerase to make it blunt ended, into Bam HI - Nru I cut Aspergillus expression vector pToC 68 (Fig. 1), and the sequence of the inserted DNA was checked (pSX 320) (cf. Fig. 2). The sequence of the full length cDNA is shown in the appended Sequence Listing ID#1.

Oligonucleotide primers used:

20 NOR 1251: 5'- AAYGCGACAAAYCC -3'
 NOR 2048: 5'- AACGAYGAYGGNAAYTTCCC -3'
 NOR 2050: 5'- AAYGAYTGGTACCAYCARTG -3'
 NOR 2153: 5'- GCGCCAGTAGCAGCCGGGCTTGAGGG -3'
 NOR 2334: 5'- ACGTCTCAACTCGGATCCAAGATGCGTT -3'
 25 Bam HI
 NOR 2378: 5'- CTCAACTCTGATCAAGATGCGTTCC -3'
 Bcl I
 NOR 2389: 5'- TGTCCAGCAGTAAGGCCCTCAAGCTG -3'
 Sal I

30 Nomenclature:

Y: Pyrimidine (C+T)
 R: Purine (A+G)
 N: All four bases

Enhanc d: Changes or insertions relative to original sequence.

Underlined: Restriction site introduced by PCR.

Expression of the ~43 kD endoglucanase:

The plasmid pSX 320 was transformed into Aspergillus oryzae A1560-T40, a protease deficient derivative of A. oryzae IFO 4177, using selection on acetamide by cotransformation with pToC 90 harboring the amdS gene from A. nidulans as a 2.7 kb Xba I fragment (Corrick et al. (1987), GENE 53, 63-71) on a pUC 19 vector (Yannisch-Perron et al. (1985), GENE 33, 103-119). Transformation was performed as described in the published EP patent application No. 238 023. A number of transformants were screened for co-expression of ~43 kD endoglucanase. Transformants were evaluated by SDS-PAGE (p.3) and CMC endoglucanase activity.

The plasmid containing the genomic gene (pCaHj 109) was transformed into Aspergillus oryzae A1560-T40 by the same procedure. Evaluation of the transformants showed that the level of expression was similar to that of the cDNA transformants.

The purified ~43 kD endoglucanase was analysed for its N-terminal sequence and carbohydrate content. The N-terminal amino acid sequence was shown to be identical to that of the HPLC purified ~43 kD endoglucanase. The carbohydrate content differs from that of the HPLC purified ~43 kD enzyme in that the recombinant enzyme contains 10 +/- 8 galactose sugars per mol rather than glucose.

Example 3

Isolation of Fusarium oxysporum genomic DNA

A freeze-dried culture of Fusarium oxysporum was reconstituted with phosphate buffer, spotted 5 times on each of 5 FOX medium plates (6% yeast extract, 1.5% K₂HPO₄, 0.75% MgSO₄ 7H₂O, 22.5% glucose, 1.5% agar, pH 5.6) and incubated at 37°C. After 6 days of incubation the colonies were scraped from the plates into 15 ml of 0.001% Tween-80 which resulted in a thick and cloudy suspension.

Four 1-liter flasks, each containing 300 ml of liquid FOX medium, were inoculated with 2 ml of the spore suspension and were incubated at 30°C and 240 rpm. On the 4th day of incubation, the cultures were filtered through 4 layers of sterile gauze and washed with sterile water. The mycelia were dried on Whatman filter paper, frozen in liquid nitrogen, ground into a fine powder in a cold mortar and added to 75 ml of fresh lysis buffer (10 mM Tris-Cl 7.4, 1% SDS, 50 mM EDTA, 100 µl DEPC). The thoroughly mixed suspension was incubated in a 65°C waterbath for 1 hour and then spun for 10 minutes at 4000 RPM and 5°C in a bench-top centrifuge. The supernatant was decanted and EtOH precipitated. After 1 hour on ice the solution was spun at 19,000 rpm for 20 minutes. The supernatant was decanted and isopropanol precipitated. Following centrifugation at 10,000 rpm for 10 minutes, the supernatant was decanted and the pellets allowed to dry.

One milliliter of TER solution (10 mM Tris-HCl, pH 7.4, 1mM EDTA 2000 100 µg RNaseA) was added to each tube, and the tubes were stored at 4°C for two days. The tubes were pooled and placed in a 65°C waterbath for 30 minutes to suspend non-dissolved DNA. The solution was extracted twice with phenol/CHCl₃/isoamyl alcohol, twice with CHCl₃/isoamyl alcohol and then ethanol precipitated. The pellet was allowed to settle and the EtOH was removed. 70% EtOH was added and the DNA was stored overnight at -20°C. After decanting and drying, 1 ml of TER was added and the DNA was dissolved by incubating the tubes at 65°C for 1 hour. The preparation yielded 1.5 mg of genomic DNA.

Cloning of Fusarium oxysporum ~43 kD endoglucanase

To isolate the Fusarium homologue to the Humicola ~43 kD cellulase PCR (as described IN US 4,683,195 and US 4,683,202) and cloned. This product was then sequenced and primers to be used as library probes and for PCR amplification were constructed. These oligonucleotides were used to isolate the corresponding clone from a cDNA library.

PCR was used to isolate partial length cDNA and genomic fragments of the 43 kD homologue. Seven different combinations of highly degenerate oligonucleotides (see table below) were used in PCR reactions with either cDNA or genomic DNA as 5 templates. Only one combination yielded partial clones of the Fusarium 43kd homologue. Two separate sets of PCR conditions were used for each oligonucleotide pair; the first set was designed to make very little product but with very high specificity. Various factors ensured specificity in this set of 10 28 cycles: The annealing temperature of 65°C was very high for these oligonucleotides; the time at annealing temperature was set for only 30 seconds; 20 picomoles of each degenerate primer mixture was used per 100 µl reaction. The oligonucleotides used contained only the degenerate region without a "cloning 15 element"; 1 unit of Amplitaq™ polymerase (Perkin-Elmer Cetus) was used per 100 µl reaction; and EDTA was added to reaction tubes at the end of the final 10 minute 72°C incubation to prevent extension from mismatched primers at cooler temperatures following the PCR cycles. Products of the first 20 set of cycles would not be expected to be visible by ethidium bromide staining in agarose gel electrophoresis due to the low efficiency of amplification required to ensure high specificity. The second set of amplifications was, however, designed to efficiently amplify products from the first set. Factors 25 ensuring this include: lowering the annealing temperature to 55°C; lengthening the time of annealing to 1 minute; increasing the amount of oligonucleotides to 100 picomoles of each mixture per 100 µl reaction; utilizing a different set of oligonucleotides which include a "Prime" cloning element along 30 with the degenerate portion (increasing the melting temperature dramatically) and by using 2.5 units of Amplitaq polymerase per 100 µl reaction.

PCR reactions were set up as recommended by Perkin-Elmer Cetus. A master mix was made for each of 2 DNA sources, genomic 35 and cDNA. This was comprised of 1X PCR buffer (10 mM Tris/HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, Perkin-Elmer Cetus), 0.2 mM deoxynucleotides (Ultrapure™ dNTP 100 mM

solution, Pharmacia), 1 unit Amplitaq™ polymerase (Perkin Elmer Cetus) and 0.5 µg genomic DNA or 50 ng cDNA per 100 µl reaction mixture volume, and deionized water to bring volume up to 98 µl per 100 µl reaction. To labeled 0.5 tubes (Eppendorf) were added 20 picomoles (1 µl of a 20 picomole/µl concentration) of each oligonucleotide mixture (see table below). These were placed in a Perkin-Elmer Cetus thermocycler at 75°C along with the master mixes and light mineral oil also in 0.5 ml tubes. Ninety eight microliters of the appropriate master mix and 55 µl light mineral oil were added to each tube with oligonucleotides. The reactions were then started in a step-cycle file (see chart below for parameters). At the end of the final 72°C incubation, 50 µl of a 10 mM EDTA pH 8.0 solution was added to each tube and incubated for a further 5 minutes at 15 72°C.

Table of oligonucleotide pairs used in 43 kD homologue PCR:

| | reaction | | oligos for first set | oligos for second set degenerate only "prime" | expected size in degenerate pairs |
|----|-----------------|-----------------|-------------------------|--|--|
| | 20 cDNA with | genomic base | | | |
| 25 | 1 | 11 | ZC3485 vs ZC3558 | ZC3486 vs ZC3559 | 288 |
| | 2 | 12 | ZC3485 vs ZC3560 | ZC3486 vs ZC3561 | 510 |
| | 3 | 13 | ZC3485 vs ZC3264 | ZC3486 vs ZC3254 | 756 |
| 30 | 4 | 14 | ZC3556 vs ZC3560 | ZC3557 vs ZC3561 | 159 |
| | 5 | 15 | ZC3556 vs ZC3264 | ZC3557 vs ZC3254 | 405 |
| 35 | 6 | 16 | ZC3556 vs ZC3465 | ZC3557 vs ZC3466 | 405 |
| | 7 | 17 | ZC3485 vs ZC3465 | ZC3486 vs ZC3466 | 756 |

Note: See oligonucleotide table for oligonucleotide sequences

Conditions for PCR step-cycle file were:

| SET 1: | | | | SET 2: | | | |
|--------|---|------|--------|--------|---|------|--------|
| | | 94°C | 1 min | | | 94°C | 1 min |
| 28 | X | 65°C | 30 sec | 28 | X | 55°C | 1 min |
| 5 | | 72°C | 2 min | | | 72°C | 2 min |
| | | 72°C | 10 min | | | 72°C | 10 min |

Following the first set of PCR cycles, DNA was purified from the reaction mixtures by isopropyl alcohol precipitation for use in the second set of cycles. Most of the light mineral oil was removed from the top of each sample before transferring the sample to a new labeled tube. Each tube was then extracted with an equal volume PCI (49% phenol: 49% chloroform: 2% isoamyl alcohol) and then with an equal volume of chloroform. DNA was then precipitated from the reactions by adding: 75 μ l 7.5 M ammonium acetate, 1 μ l glycogen and 226 μ l isopropyl alcohol. Pellets were resuspended in 20 μ l deionized water. Two microliters of each resuspension were placed into labeled tubes for the second round of PCR amplifications along with 100 picomoles (5 μ l of a 20 picomole/ μ l concentration) of each new primer mixture (see table above). A master mix was made as described above except for excluding Alegenomic and cDNA templates and compensating for increased oligonucleotide and DNA volumes in the reaction tubes by decreasing the volume of water added. Reactions and cycles were set up as described above (see table above).

After the 28 cycles were completed, light mineral oil was removed from the tops of the samples, and the PCR mixtures were removed to new tubes. Ten microliters of each sample were spotted onto parafilm and incubated at 45°C for approximately 5 minutes to allow the sample to decrease in volume and to allow the parafilm to absorb any residual light mineral oil. The drops were then combined with 2 μ l 6X loading dye and electrophoresed on 1% agarose (Seakem GTG™, FMC, Rockland, ME) gel. A single band of approximately 550 base pairs was found in

reaction number 2 where the template was cDNA. A band of approximately 620 base pairs in reaction number 12 where the template was genomic DNA. These reactions were primed with oligonucleotides ZC3486 and ZC3561 (Table 1). This was very close to the 510 base pair PCR product predicted from comparison with the Humicola 43kD sequence. The synthesis of a larger product in the reaction with genomic template is due to the presence of an intron within this region. The agarose containing these 2 bands was excised and DNA was extracted utilizing a Prep-A-Gene™ kit (BioRad) following manufacturers instructions. DNA was eluted with 50 µl deionized water and precipitated with 5 µl 3M sodium acetate, 1 µl glycogen and 140 µl ethanol. The DNA pellet was dried and resuspended in a volume of 7 µl TE (10 mM Tris-HCL pH 8.0, 1mM EDTA).

15 The PCR fragments were cloned into pBS sk-'vector was constructed by first digesting pBluescript II sk- (Stratagene, La Jolla, CA) with Eco RI and gel purifying cut plasmid from 0.8% seaplaque GTG™ agarose (FMC) with a Pre-A-Gene™ kit (BioRad) following the manufacturer's instructions.

20 Oligonucleotides ZC1773 and ZC1774 (Table 1) were annealed by mixing 2 picomoles of each oligonucleotide, bringing up the reaction volume to 4 µl with deionized water then adding 0.5 µl annealing buffer (200mM Tris-HCl pH 7.6, 50mM MgCl₂) and bringing the temperature up to 65°C for 30 seconds and slowly cooling to 25 20°C in 20 minutes in a Perkin-Elmer Cetus PCR thermocycler. The oligonucleotides were then ligated into the Eco RI digested pBluescript vector by mixing: 5.5 µl deionized water, 2µl annealed oligonucleotides, 1µl of a 1:3 dilution in deionized water of digested vector, 1µl 10X T4 DNA ligase buffer 30 (Boehringer-Mannheim Biochemicals, Indianapolis IN) and 0.5 T4 DNA ligase (Gibco-BRL), and incubating the mixture at 16°C for 2.5 hours. The ligation mixture was then brought up to a volume of 100 µl with deionized water and extracted with PCI and chloroform. To increase electroporation efficiency, DNA was 35 then precipitated with 50 µl ammonium acetate, 1 µl glycogen and 151 µl isopropanol. One microliter of a 10 µl resuspension in deionized water was electroporated into E. coli DH10-B

electromax cells (Gibco-BRL) using manufacturer's instructions, in a Bio-Rad electroporation apparatus. Immediately following the electroporation, 1 ml of 2XYT (per liter: 16 g tryptone, 10 g yeast extract, 10 g NaCl) broth was added to the cuvet and 5 mixed. Various dilutions were plated onto 100 mm LB plates (per liter: 10 g tryptone, 8 g yeast extract, 5 g NaCl, 14.5 g agar) with 100 µg/ml ampicillin, and coated with 100 µl of 20 mg/ml X-Gal (5-Bromo-4 Chloro-3-Indolyl-b-D-galactopyranoside; Sigma, St. Louis, MO) in dimethylformamide and 20 µl of 1M IPTG 10 (Sigma). After overnight growth various blue and white colonies were analyzed by PCR for small inserts using the oligonucleotides ZC3424 (bluescript reverse primer) and ZC3425 (T7 promoter primer) (Table 1), following conditions outlined above for screening bacterial plugs. After an initial 1 minute 15 45 seconds at 94°C denaturation, 30 cycles of 94°C for 45 seconds, 40° for 30 seconds and 72°C for 1 minute were performed. Upon agarose gel electrophoresis of the PCR products, 1 blue colony giving a PCR band consistant with a small insert in the pBluescript cloning region was chosen for 20 DNA purification and was grown up overnight in a 100 ml liquid culture in TB (per liter: 12 g tryptone, 24 g yeast extract, 4 ml glycerol, autoclave. Then add 100 ml of 0.17M KH₂PO₄, 0.72M K₂HPO₄; Sambrook et al., Molecular Cloning, 2nd Ed., 1989, A.2) with 150 µg/ml ampicillin. DNA was isolated by alkaline lysis 25 and PEG precipitation (Sambrook et al., Molecular Cloning 2nd ed., 1.38-1.41, 1989). Sequence analysis showed the correct oligonucleotide to be inserted while maintaining the β-galactosidase gene present in pBluescript vectors in frame with the promoter. Fifty micrograms of the DNA preparation was 30 digested with Eco RI, PCI and chloroform extracted, and precipitated with sodium acetate and ethanol. The DNA pellet was resuspended in 50 µl deionized water. Digested pBS sk-' was cut back with T4 DNA polymerase (Gibco-BRL) by adding 40 µl 10 X T4 DNA polymerase buffer (0.33M Tri/acetate pH 8.0, 0.66M 35 potassium acetate, 0.1M magnesium acetate, 5mM dithiotheretiol, 5mM BSA (New England Biolabs) 260 µl deionized water, 40 µl 1mM dTTP (Ultrapure™, Pharmacia) and 40 µl T4 DNA polymerase (1

U/ μ l) (Gibco-BRL) to 20 μ l of 1mg/ml vector DNA. The mixture was incubated at 12°C for 15 minutes, then at 75°C for 10 minutes. To prepare the DNA for use in ligation, it was PCI and chloroform extracted and precipitated with sodium acetate and 5 ethanol. The pellet was resuspended in 200 μ l deionized water, producing a concentration of 0.1 μ g/ μ l.

To prepare the 43kd homologue PCR products for insertion into the cut-back pBS sk-' vector, they were cut back with T4 DNA polymerase (Gibco-BRL) in reaction volumes of 10 μ l with 10 the inclusion of dATP instead of dTTP. The resulting DNA solutions were PCI and chloroform extracted and precipitated with sodium acetate, glycogen and ethanol. The DNA pellets were resuspended in 15 μ l deionized water. DNA samples of 7.5 μ l were ligated into 0.1 μ g cut back pBS sk-' (0.1 μ g/ μ l) with 1 15 μ l 10X ligase buffer (Boehringer-Mannheim) and 0.5 μ l of T₄ DNA ligase (Boehringer-Mannheim). The ligation mixtures were then brought up to a volume of 150 μ l with deionized water and extracted with PCI and chloroform. To increase electroporatoin efficiency, DNA was then precipitated with 15 μ l sodium 20 acetate, 1 μ l glycogen and 166 μ l isopropanol. One microliter of a 10 μ l resuspension in deionized water was electroparated into E. coli DH10-B electromax cells (BRL) using a Bio-Rad electroporation apparatus, according to manufacturer's instructions. Immediately following the electroporation, 1 ml 25 of SOB broth (per liter: 20 g tryptone, 5 g yeast extract, 10 ml 1M NaCl, 2.5 ml 1M KCl. Autoclave then add 10 ml 1 M MgCl₂ and 10 ml 1M MgSO₄) was added to the cuvet, and the cell mixture was transferred to a 100 mm tube and incubated at 37°C for 1 hour with aeration. Various dilutions were plated onto 100 mm 30 LB plates containing 100 μ g/ml ampicillin and coated with 100 μ l of 20 mg/ml X-Gal (Sigma) in dimethylformamide and 20 μ l of 1M IPTG (Sigma). Three white colonies of each of the 2 transformations, cDNA and genomic, were picked for sequencing. Sequence analysis showed the inserts to be highly homologous to 35 the Humicola 43 kDcellulase. The genomic insert was identical to the cDNA except for the presence of an intron. Two 42-mer ologonucleotides ZC3709 and ZC3710 (Table 1) were designed from

the sequence for use as library probes and PCR primers. The oligonucleotides were from opposite ends of the PCR product and were designed to hybridize opposite strands of the DNA so that they could be used as primers in a PCR reaction to test 5 potential clones in the library screening.

Construction of a Fusarium oxysporum cDNA library

Fusarium oxysporum was grown by fermentation and samples were withdrawn at various times for RNA extraction and cellulase activity analysis. The activity analysis included an assay for 10 total cellulase activity as well as one for colour clarification. Fusarium oxysporum samples demonstrating maximal colour clarification were extracted for total RNA from which poly(A)+RNA was isolated.

To construct a Fusarium oxysporum cDNA library, first-strand 15 cDNA was synthesized in two reactions, one with and the other without radiolabelled dATP. A 2.5X reaction mixture was prepared at room temperature by mixing the following reagents in the following order: 10 μ l of 5X reverse transcriptase buffer (Gibco-BRL, Gaithersburg, Maryland) 2.5 μ l 200 mM 20 dithiothreitol (made fresh or from a stock solution stored at -70°C), and 2.5 μ l of a mixture containing 10 mM of each deoxynucleotide triphosphate, (dATP, dGTP, dTTP and 5-methyl dCTP, obtained from Pharmacia LKB Biotechnology, Alameda, CA). The reaction mixture was divided into each of two tubes of 7.5 25 μ l. 1.3 μ l of 10 μ Ci/ μ l 32 P α -dATP (Amersham, Arlington Heights, IL) was added to one tube and 1.3 μ l of water to the other. Seven microliters of each mixture was transferred to final reaction tubes. In a separate tube, 5 μ g of Fusarium oxysporum poly (A)⁺ RNA in 14 μ l of 5 mM Tris-HCl pH 7.4, 50 μ M 30 EDTA was mixed with 2 μ l of 1 μ g/ μ l first strand primer (ZC2938 GACAGAGCACAGAATTCACTAGTGAGCTCT₁₅). The RNA-primer mixture was heated at 65°C for 4 minutes, chilled in ice water, and centrifuged briefly in a microfuge. Eight microliters of the RNA-primer mixture was added to the final reaction tubes. Five

microliters of 200 U/ μ l SuperscriptTM reverse transcriptase (Gibco-BRL) was added to each tube. After gentle agitation, the tubes were incubated at 45°C for 30 minutes. Eighty microliters of 10 mM Tris-HCl pH 7.4, 1 mM EDTA was added to each tube, the 5 samples were vortexed, and briefly centrifuged. Three microliters was removed from each tube to determine counts incorporated by TCA precipitation and the total counts in the reaction. A 2 μ l sample from each tube was analyzed by gel electrophoresis. The remainder of each sample was ethanol 10 precipitated in the presence of oyster glycogen. The nucleic acids were pelleted by centrifugation, and the pellets were washed with 80% ethanol. Following the ethanol wash, the samples were air dried for 10 minutes. The first strand synthesis yielded 1.6 μ g of Fusarium oxysporum cDNA, a 33% 15 conversion of poly(A)+RNA into DNA.

Second strand cDNA synthesis was performed on the RNA-DNA hybrid from the first strand reactions under conditions which encouraged first strand priming of second strand synthesis resulting in hairpin DNA. The first strand products from each 20 of the two first strand reactions were resuspended in 71 μ l of water. The following reagents were added, at room temperature, to the reaction tubes: 20 μ l of 5X second strand buffer (100 mM Tris pH 7.4, 450 mM KCl, 23 mM MgCl₂, and 50 mM (NH₄)₂(SO₄), 3 μ l of 5 mM β -NAD, and μ l of a deoxynucleotide triphosphate 25 mixture with each at 10 mM. One microliter of α -³²P dATP was added to the reaction mixture which received unlabeled dATP for the first strand synthesis while the tube which received labeled dATP for first strand synthesis received 1 μ l of water. Each tube then received 0.6 μ l of 7 U/ μ l E. coli DNA ligase 30 (Boehringer-Mannheim, Indianapolis, IN), 3.1 μ l of 8 U/ μ l E. coli DNA polymerase I (Amersham), and 1 μ l 2 U/ μ l of RNase H (Gibco-BRL). The reactions were incubated at 16°C for 2 hours. After incubation, 2 μ l from each reaction was used to determine TCA precipitable counts and total counts in the reaction, and 35 2 μ l from each reaction was analyzed by gel electrophoresis. To the remainder of each sample, 2 μ l of 2.5 μ g/ μ l oyster

glycogen, 5 μ l of 0.5 EDTA and 200 μ l of 10 mM Tris-HCl pH 7.4, 1 mM EDTA were added. The samples were phenol-chloroform extracted and isopropanol precipitated. After centrifugation the pellets were washed with 100 μ l of 80% ethanol and air dried. The yield of double stranded cDNA in each of the reactions was approximately 2.5 μ g.

Mung bean nuclease treatment was used to clip the single-stranded DNA of the hair-pin. Each cDNA pellet was resuspended in 15 μ l of water and 2.5 μ l of 10X mung bean buffer (0.3 M NaAc pH 4.6, 3 M NaCl, and 10 mM ZnSO₄), 2.5 μ l of 10 mM DTT, 2.5 μ l of 50% glycerol, and 2.5 μ l of 10 U/ μ l mung bean nuclease (New England Biolabs, Beverly, MA) were added to each tube. The reactions were incubated at 30°C for 30 minutes and 75 μ l of 10 mM Tris-HCl pH 7.4 and 1 mM EDTA was added to each tube. Two-microliter aliquots were analyzed by alkaline agarose gel analysis. One hundred microliters of 1 M Tris-HCl pH 7.4 was added to each tube and the samples were phenol-chloroform extracted twice. The DNA was isopropanol precipitated and pelleted by centrifugation. After centrifugation, the DNA pellet was washed with 80% ethanol and air dried. The yield was approximately 2 μ g of DNA from each of the two reactions.

The cDNA ends were blunted by treatment with T4 DNA polymerase. DNA from the two samples were combined after resuspension in a total volume of 24 μ l of water. Four microliters of 10X T4 buffer (330 mM Tris-acetate pH 7.9, 670 mM KAc, 100 mM MgAc, and 1 mg/ml gelatin), 4 μ l of 1 mM dNTP, 4 μ l 50 mM DTT, and 4 μ l of 1 U/ μ l T4 DNA polymerase (Boehringer-Mannheim) were added to the DNA. The samples were incubated at 15°C for 1 hour. After incubation, 160 μ l of 10 mM Tris-HCl pH 7.4, 1 mM EDTA was added, and the sample was phenol-chloroform extracted. The DNA was isopropanol precipitated and pelleted by centrifugation. After centrifugation the DNA was washed with 80% ethanol and air dried.

After resuspension of the DNA in 6.5 μ l water, Eco RI adapters were added to the blunted DNA. One microliter of 1 μ g/ μ l Eco RI adapter (Invitrogen, San Diego, CA Cat. # N409-20), 1 μ l of 10X ligase buffer (0.5 M Tris pH 7.8 and 50 mM $MgCl_2$), 0.5 μ l of 10 mM ATP, 0.5 μ l of 100 mM DTT, and 1 μ l of 1 U/ μ l T4 DNA ligase (Boehringer-Mannheim) were added to the DNA. After the sample was incubated overnight at room temperature, the ligase was heat denatured at 65°C for 15 minutes.

The Sst I cloning site encoded by the first strand primer was exposed by digestion with Sst I endonuclease. Thirty-three microliters of water, 5 μ l of 10X Sst I buffer (0.5 M Tris pH 8.0, 0.1 M $MgCl_2$, and 0.5 M NaCl), and 2 μ l of 5 U/ μ l Sst I were added to the DNA, and the samples were incubated at 37°C for 2 hours. One hundred and fifty microliters of 10 mM Tris-HCl pH 7.4, 1 mM EDTA was added, the sample was phenol-chloroform extracted, and the DNA was isopropanol precipitated.

The cDNA was chromatographed on a Sepharose CL 2B (Pharmacia LKB Biotechnology) column to size-select the cDNA and to remove free adapters. A 1.1 ml column of Sepharose CL 2B was poured into a 1 ml plastic disposable pipet and the column was washed with 50 column volumes of buffer (10 mM Tris pH 7.4 and 1 mM EDTA). The sample was applied, one-drop fractions were collected, and the DNA in the void volume was pooled. The fractionated DNA was isopropanol precipitated. After centrifugation the DNA was washed with 80% ethanol and air dried.

A Fusarium oxysporum cDNA library was established by ligating the cDNA to the vector pYcDE8' (cf. WO 90/10698) which had been digested with Eco RI and Sst I. Three hundred and ninety nanograms of vector was ligated to 400 ng of cDNA in a 80 μ l ligation reaction containing 8 μ l of 10 X ligase buffer, 4 μ l of 10 mM ATP, 4 μ l 200 mM DTT, and 1 unit of T4 DNA ligase (Boehringer-Mannheim. After overnight incubation at room temperature, 5 μ g of oyster glycogen and 120 μ l of 10 mM Tris-

HCl and 1 mM EDTA were added and the sample was phenol-chloroform extract d. The DNA was ethanol precipitated, centrifuged, and the DNA pellet washed with 80% ethanol. After air drying, the DNA was resuspended in 3 μ l of water. Thirty
5 seven microliters of electroporation competent DH10B cells (Gibco-BRL) was added to the DNA, and electroporation was completed with a Bio-Rad Gene Pulser (Model #1652076) and Bio-Rad Pulse Controller (Model #1652098) electroporation unit (Bio-Rad Laboratories, Richmond, CA). Four milliliters of SOC
10 (Hanahan, J. Mol. Biol. 166 (1983), 557-580) was added to the electroporated cells, and 400 μ l of the cell suspension was spread on each of ten 150 mm LB ampicillin plates. After an overnight incubation, 10 ml of LB amp media was added to each plate, and the cells were scraped into the media. Glycerol
15 stocks and plasmid preparations were made from each plate. The library background (vector without insert) was established at approximately 1% by ligating the vector without insert and titering the number of clones after electroporation.

To isolate full length cDNA clones of the 43 kD homologue a
20 library of 1,100,000 clones was plated out onto 150 mm LB plates with 100 μ g/ml ampicillin. One hundred thousand clones were plated out from glycerol stocks onto each of 10 plates and 20,000 clones were plated out on each of 5 plates. Lifts were taken in duplicate as described above. Prehybridization,
25 hybridization and washing were also carried out as described above. Two end labeled 42-mer oligonucleotides, ZC3709 and ZC3710 (which are specific for the 43kD homologue), were used in the hybridization. Filters were washed once for 20 minutes with TMACl at 77°C. Twenty two spots showing up on duplicate
30 filters were found. Corresponding areas on the plates were picked with the large end of a pipet into 1 ml of 1 X PCR buffer. These isolated analyses by PCR were with 2 sets of oligonucleotides for each isolate. One set contained the two 43
kD specific oligonucleotides used as hybridization probes and
35 the other contained one 43 kD specific oligonucleotide, ZC3709, and one vector specific oligonucleotide, ZC3634. PCR was

conduct d as before by Perkin Elmer Cetus directions. Twenty picomol s of each primer and 5 μ l of the cell suspension were used in each reaction of 50 μ l. After an initial 1 minute 30 second denaturation at 94°C 30 cycles of 1 minute at 94°C and 5 2 minutes at 72°C were employed, with a final extension time of 10 minutes at 72°C. Results showed 17 of the 22 to contain the 2 43 kD specific oligonucleotide recognition sites. The remaining 5 clones contained one of the 2 sites, ZC3709, but were shown by PCR with the vector specific primer to be 10 truncated and not long enough to contain the other site. The 9 longest clones were chosen for single colony isolation through another level of screening. Five 10 fold dilutions of each were plated out and processed as described above for the first set of lifts. All of the nine had signals on autoradiograms of the 15 second level of screening. Colonies were fairly congested so a few separate colonies in the area of the radioactive signal were single colony isolated on 150 mm LB plates with 70 μ g/ml ampicillin. These were tested by PCR for homologues to the ~43 kD endoglucanase with the oligonucleotides ZC3709 and ZC3710 as 20 described for the first level of screening except that colonies were picked by toothpick into 25 μ l of mastermix. Bands of the expected size were obtained for 7 of the 9 clones. Cultures of these were started in 20 ml of Terrific Broth with 150 μ g/ml ampicillin. DNA was isolated by alkaline lysis and PEG 25 precipitation as above.

DNA sequence analysis

The cDNAs were sequenced in the yeast expression vector pYCDE8'. The dideoxy chain termination method (F. Sanger et al., Proc. Natl. Acad. Sci. USA 74, 1977, pp. 5463-5467) using 30 @35-S dATP from New England Nuclear (cf. M.D. Biggin et al., Proc. Natl. Acad. Sci. USA 80, 1983, pp. 3963-3965) was used for all sequencing reactions. The reactions were catalysed by modified t7 DNA polymerase from Pharmacia (cf. S. Tabor and C.C. Richardson, Proc. Natl. Acad. Sci. USA 84, 1987, pp. 4767- 35 4771) and were primed with an oligonucleotide complementary to

the ADH1 promoter (ZC996: ATT GTT CTC GTT CCC TTT CTT), complementary to the CYC1 terminator (ZC3635: TGT ACG CAT GTA ACA TTA) or with oligonucleotides complementary to the DNA of interest. Double stranded templates were denatured with NaOH 5 (E.Y. Chen and P.H. Seeburg, DNA 4, 1985, pp. 165-170) prior to hybridizing with a sequencing oligonucleotide. Oligonucleotides were synthesized on an Applied Biosystems Model 380A DNA synthesizer. The oligonucleotides used for the sequencing reactions are listed in the sequencing oligonucleotide table 10 below:

Table 1:

Oligonucleotides for 43 kD homologue PCR:

ZC3485 TGG GA(C/T) TG(C/T) TG(C/T) AA(A/G) CC
 ZC3486 AGG GAG ACC GGA ATT CTG GGA (C/T)TG (C/T)TG (C/T)
 15 AA(A/G) CC
 ZC3556 CC(A/C/G/T) GG(A/C/G/T) GG(A/C/G/T) GG(A/C/G/T)
 GT(A/C/G/T) GG
 ZC3557 AGG GAG ACC GGA ATT CCC (A/C/G/T)GG (A/C/G/T)GG
 (A/C/G/T)GG (A/C/G/T)GT (A/C/G/T)GG
 20 ZC3558 AC(A/C/G/T) A(C/T)C AT(A/C/G/T) (G/T)T/C/T) TT(A/C/G/T)
 CC
 ZC3559 GAC AGA GCA CAG AAT TCA C(A/C/G/T)A (C/T)CA
 T(A/C/G/T)(G/T) T(C/T)T T(A/C/G/T)C C
 ZC3560 (A/C/G/T)GG (A/G)TT (A/G)TC (A/C/G/T)GC
 25 (A/C/G/T)(G/T)(C/T) (C/T)T(C/T) (A/G)AA CCA
 ZC3561 GAC AGA GCA CAG AAT TC(A/C/G/T) GG(A/G) TT(A/G)
 TC(A/C/G/T) GC(A/C/G/T) (G/T)(C/T)(C/T) T(C/T)(A/G)
 AAC CA

Oligonucleotides for 43 kD homologue cloning:

30 ZC3709 GGG GTA GCT ATC ACA TTC GCT TCG GGA GGA GAT ACC GCC
 GTA
 ZC3710 CTT CTT GCT CTT GGA GCG GAA AGG CTG CTG TCA ACG CCC
 CTG

pYCDE8' vector oligonucleotides:

ZC3635 TGT ACG CAT GTA ACA TTA CYC 1 terminator

ZC3634 CTG CAC AAT ATT TCA AGC ADH 1 promoter

43kD homologue specific sequencing primers:

5 ZC3709 GGG GTA GCT ATC ACA TTC GCT TCG GGA GGA GAT ACC GCC GTA

ZC3710 CTT CTT GCT CTT GGA GCG GAA AGG CTG CTG TCA ACG CCC CTG

ZC3870 AGC TTC TCA AGG ACG GTT

ZC3881 AAC AAG GGT CGA ACA CTT

ZC3882 CCA GAA GAC CAA GGA TT

10 Example 4**Colour clarification test**

The Humicola ~43 kD endoglucanase (a mixture of 30 purification runs) was compared in a colour clarification test with the H. insolens cellulase preparation described in US 15 4,435,307, Example 6.

Old worn black cotton swatches are used as the test material. The clarification test is made in a Terg-O-tometer making three repeated washes. Between each wash the swatches are dried overnight.

20 Conditions:

2 g/l of liquid detergent at 40°C for 30 min. and a water hardness of 9°dH. The swatch size is 10x15 cm, and there are two swatches in each beaker.

The composition of the detergent was as follows:

25 10% anionic surfactant (Nansa 1169/p)

15% non-ionic surfactant (Berol 160)

10% ethanol

5% triethanol amine

60% water

30 pH adjusted to 8.0 with HCl.

Dosage:

The two enzymes are dosed in 63 and 125 CMC-endoase units/l.

Results:

5 The results were evaluated by a panel of 22 persons who rated the swatches on a scale from 1 to 7 points. The higher the score, the more colour clarification obtained.

| | Enzyme | CMC-endoase/l | Protein mg/l | PSU* |
|----|-------------------------------------|---------------|--------------|------------------------|
| 10 | No enzyme | | | 1.4 ± 1.0 |
| 15 | H. insolens cellulase mixture | 63 125 | 14 28 | 5.8 ± 1.0 6.1 ± 1.0 |
| 20 | Invention | 63 125 | 0.4 0.8 | 4.6 ± 0.9 6.2 ± 0.8 |

25 * PSU = Panel Score Units

The ~43 kD endoglucanase is shown to have an about 30 times better performance than the prior art H. insolens cellulase mixture and an about 6 times better performance than the cellulase preparation according to WO 89/09259.

30 Example 5

Stability of the Humicola ~43 kD endoglucanase in the presence of proteases

The storage stability of the ~43 kD endoglucanase in liquid detergent in the presence of different proteases was 35 determined under the following conditions:

Enzymes

- ~43 kD endoglucanase of the invention
Glu/Asp specific B. licheniformis serine protease
Trypsin-like Fusarium sp. DSM 2672 protease
5 B. lentus serine protease
Subtilisin Novo

Detergent

US commercial liquid detergent not containing any opacifier, perfume or enzymes (apart from those added in the 10 experiment). +/- 1% (w/w) boric acid as enzyme stabiliser.

Dosage

| | |
|----------------|------------------------|
| Endoglucanase: | 12 CMCU/g of detergent |
| Proteases: | 0.2 mg/g of detergent |

Incubation

- 15 7 days at 35°C

Residual activity

The residual activity of the endoglucanase after 7 days of incubation with the respective proteases was determined in terms of its CMCase activity (CMCU).

- 20 The CMCase activity was determined as follows:

A substrate solution of 30 g/l CMC (Hercules 7 LFD) in deionized water was prepared. The enzyme sample to be determined was dissolved in 0.01 M phosphate buffer, pH 7.5. 1.0 ml of the enzyme solution and 2.0 ml of a 0.1 M phosphate 25 buffer, pH 7.5, were mixed in a test tube, and an enzyme reaction was initiated by adding 1.0 ml of the substrate solution to the test tube. The mixture was incubated at 40°C for 20 minutes, after which the reaction was stopped by adding 2.0 ml of 0.125 M trisodium phosphate.12H₂O. A blind sample was 30 prepared without incubation.

2.0 ml of a ferricyanide solution (1.60 g of potassium ferricyanide and 14.0 g of trisodium phosphate.12H₂O in 1 l of deionized water) was added to a test sample as well as to a blind immediately followed by immersion in boiling water and 5 incubation for 10 minutes. After incubation, the samples were cooled with tap water. The absorbance at 420 nm was measured, and a standard curve was prepared with glucose solution.

One CMCase unit (CMCU) is defined as the amount of enzyme which, under the conditions specified above, forms an 10 amount of reducing carbohydrates corresponding to 1 μ mol of glucose per minute.

Results

The storage stability of the endoglucanase of the invention was determined as its residual activity (in CMCU%) 15 under the conditions indicated above.

| Protease | | Residual Activity (%) | |
|----------|-------------------------|-----------------------|--------------|
| | | + boric acid | - boric acid |
| 20 | Glu/Asp specific | 105 | 93 |
| | Trypsin-like | 77 | 63 |
| | <u>B. lentus</u> serine | 57 | 24 |
| | Subtilisin Novo | 63 | 55 |

These results indicate that the storage stability in liquid 25 detergent of the endoglucanase of the invention is improved when a protease with a higher degree of specificity than Savinase[®] is included in the detergent composition.

Example 6

Use of Humicola ~43 kD endoglucanase to provide a localized 30 variation in colour of denim fabric

Denim jeans were subjected to treatment with the ~43 kD endoglucanase in a 12 kg "Wascator" FL120 wash extractor with

a view to imparting a localized variation in the surface colour of the jeans approximating a "ston washed" appearance.

Four pairs of jeans were used per machine load. The experimental conditions were as follows.

5 Desizing

40 l water
100 ml B. amyloliquefaciens amylase*, 120 L
70 g KH_2PO_4
30 g Na_2HPO_4
10 55°C
10 minutes
pH 6.8

*available from Novo Nordisk A/S.

The desizing process was followed by draining.

15 Abrasion

40 l water
120 g H. insolens cellulase mixture or
x g ~43 kD endoglucanase
70 g KH_2PO_4
20 30 g Na_2HPO_4
55°C
75 minutes
pH 6.6

The abrasion process was followed by draining, rinsing, after-25 washing and rinsing.

The results were evaluated by judging the visual appearance of the jeans.

Different dosages of ~43 kD endoglucanase were used to obtain an abrasion level which was equivalent to that obtained 30 with 120 g H. insolens cellulase mixture. Such an equivalent level was obtained with 1.0-1.25 g of ~43 kD endoglucanase. Measurements of the tear strength of the treated garments showed no significant difference between the two enzyme treatments.

Example 7**Use of Humicola ~43 kD endoglucanase to remove fuzz from fabric surface**

Woven, 100% cotton fabric was treated with the ~43 kD endoglucanase in a 12 kg "Wascator" FL120 wash extractor with a view to investigating the ability of the enzyme to impart a greater degree of softness to new fabric.

The experimental conditions were as follows.

Fabric

- 10 Woven, 100% cotton fabric obtained from Nordisk Textil, bleached (NT2116-b) or unbleached (NT2116-ub). 400 g of fabric were used per machine load.

Desizing

- 40 l water
15 200 ml B. amyloliquefaciens amylase, 120 L
60 g KH_2PO_4
20 g Na_2HPO_4
60°C
10 minutes
20 pH 6.4

The desizing process was followed by draining.

Main wash

- 40 l water
0-600 g H. insolens cellulase mixture or
25 x g ~43 kD endoglucanase
60 g KH_2PO_4
40 g Na_2HPO_4
60°C
60 minutes
30 pH 6.7

The abrasion step was followed by draining.

Afterwash

40 l water
40 g Na_2CO_3
10 g Berol 08
5 80°C
15 minutes
pH 10.1

The afterwash was followed rinsing.

Three different concentrations of the 43 kD
10 endoglucanase were added in the main wash.

The weight loss of the fabric samples was measured before and after treatment. The weight loss is expressed in % and is related to the desized fabric.

Fabric thickness was measured by means of a thickness
15 measurer L&W, type 22/1. 2 swatches of the fabric (10 x 6 cm) were measured, and 5 measurements in μm were recorded for each swatch. The swatch was measured at a pressure of 98.07 kPa. The retained thickness is expressed in % in relation to the desized fabric.

20 Fabric strength was measured by means of a tearing tester (Elmendorf 09). 6 swatches (10 x 6 cm) were cut in the warp direction and 6 swatches (10 x 6 cm) in the weft direction. The tear strength was measured in mN in accordance with ASTM D 1424. The fabric strength of the enzyme-treated
25 fabric is expressed in % in relation to the desized fabric.

Fabric stiffness was measured by means of a King Fabric Stiffness Tester. 4 swatches (10 x 20 cm; 10 cm in the warp direction) are cut from the fabric, and each swatch is folded back to back (10 x 10 cm) and placed on a table provided with
30 an open ring in the middle. A piston pushes the fabric through the ring using a certain power expressed in grammes. The determination is made according to the ASTM D 4032 Circular Bend Test Method. Retained fabric stiffness is expressed in % in relation to the desized fabric.

The results of these tests appear from the following table:

| | | | | | |
|----|------------------------|------------------|-------------------------|------------------------|-------------------------|
| 5 | Enzyme Dosage EUG/1 | Weight Loss % | Retained Thickness % | Retained Strength % | Retained Stiffness % |
| | 0 | 0 | 100 | 100 | 100 |
| | 13 | 4.0 | 95.3 | 85.4 | 88.6 |
| 10 | 50 | 5.1 | 94.5 | 73.3 | 85.0 |
| | 150 | 7.7 | 91.9 | 70.7 | 79.3 |

Example 8

Use of Humicola ~43 kD endoglucanase for the treatment of paper pulp

The ~43 kD endoglucanase was used for the treatment of several types of paper pulp with a view to investigating the effect of the enzyme on pulp drainage.

The experimental conditions were as follows.

20 Pulps

1. Waste paper mixture: composed of 33% newsprint, 33% magazines and 33% computer paper. With or without deinking chemicals (WPC or WP, respectively)
2. Recycled cardboard containers (RCC).
- 25 3. Bleached kraft: made from pine (BK).
4. Unbleached thermomechanical: made from fir (TMP).

Determination of cellulase activity (CEVU)

A substrate solution containing 33.3 g/l CMC (Hercules 7 LFD) in Tris-buffer, pH 9.0, is prepared. The enzyme sample to be determined is dissolved in the same buffer. 10 ml substrate solution and 0.5 ml enzyme solution are mixed and transferred to a viscosimeter (Haake VT 181, NV sensor, 181 rpm) thermostated at 40°C. One Cellulase Viscosity Unit (CEVU) is defined in Novo Nordisk Analytical Method No. AF 253 (available from Novo Nordisk).

Determination of pulp drainage (Schopper-Riegler)

The Schopper-Riegler number (SR) is determined according to ISO standard 5267 (part 1) on a homogenous pulp with a consistency of 2 g/l. A known volume of pulp is allowed to drain through a metal sieve into a funnel. The funnel is provided with an axial hole and a side hole. The volume of filtrate that passes through the side hole is measured in a vessel graduated in Schopper-Riegler units.

Enzymatic treatment

10 A preparation of the 43 kD endoglucanase was diluted to 7 CEVU/ml and added to each of the pulps indicated above (50 g DS, consistency 3%). The enzyme dose was 2400 CEVU/kg dry pulp. The enzymatic treatment was conducted at a pH of 7.5 and at 40°C with gentle stirring for 60 minutes. A sample was taken 15 after 30 minutes to monitor the progression of the reaction. After 60 minutes, the pulp was diluted to a consistency of 0.5% with cold water (+4°C) in order to stop the reaction.

Drainage of the wet pulp was determined as described above and assigned Schopper-Riegler (SR) values. The drainage 20 time (DT) under vacuum was also determined.

The results are summarized in the following table.

| 25 | | Waste paper + chemicals | |
|----|---|-------------------------|--------|
| | | Control | Enzyme |
| | SR (3%) | 61 | 55 |
| 30 | Drainage time (s) 150 g/m ² | 18.2 | 17 |
| | Mass g/m ² | 65.6 | 66.4 |
| | Vol cm ³ /g | 1.65 | 1.66 |
| 35 | Breaking Length, m | 3650 | 3970 |
| | Burst Index | 2.19 | 2.47 |

| | | Waste paper | |
|----|---|-------------|--------|
| | | Control | Enzyme |
| 5 | SR (3%) | 59 | 51 |
| | Drainage time (s) 150 g/m ² | 18.2 | 12.7 |
| 10 | Mass g/m ² | 68.0 | 67.9 |
| | Vol cm ³ /g | 1.68 | 1.64 |
| 15 | Breaking Length, m | 3810 | 3790 |
| | Burst Index | 2.25 | 2.33 |

| | | Recycled Cardboard Containers | |
|----|---|-------------------------------|--------|
| | | Control | Enzyme |
| 20 | SR (3%) | 45 | 33 |
| | Drainage time (s) 150 g/m ² | 6.8 | 5.3 |
| 25 | Mass g/m ² | 70.2 | 67.3 |
| | Vol cm ³ /g | 1.91 | 1.99 |
| 30 | Breaking Length, m | 3640 | 3530 |
| 35 | Burst Index | 2.25 | 2.22 |

| | | Kraft | |
|----|---|---------|--------|
| | | Control | Enzyme |
| 5 | SR (3%) | 42 | 31 |
| | Drainage time (s) 150 g/m ² | 10.7 | 6 |
| 10 | Mass g/m ² | 67.5 | 69,1 |
| | Vol cm ³ /g | 1.44 | 1.42 |
| 15 | Breaking Length, m | 7010 | 7190 |
| | Burst Index | 5.14 | 4.96 |

| | | TMP | |
|----|---|---------|--------|
| | | Control | Enzyme |
| 20 | SR (3%) | 68 | 60 |
| | Drainage time (s) 150 g/m ² | 13.8 | 11.3 |
| 25 | Mass g/m ² | 68.7 | 70.2 |
| | Vol cm ³ /g | 2.13 | 2.04 |
| 30 | Breaking Length, m | 3630 | 3620 |
| 35 | Burst Index | 1.95 | 1.91 |

Tabel 3: Results of the drainage and strength measurements.

Control experiments. Same conditions as the enzyme treatment,

It appears from the table that the ~43 kD endoglucanase treatment causes a significant decrease in SR values and significantly improves drainage of pulps used in papermaking.

Paper sheets were made from the various pulps on a Rapid Kōthen device and measured for strength according to different parameters (including breaking length). No decrease in strength properties due to enzyme action was observed.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- 5 (i) APPLICANT: NOVO NORDISK A/S, N N
- (ii) TITLE OF INVENTION: A Cellulase Preparation
- 10 (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: NOVO NORDISK A/S, Patent Department
- (B) STREET: Novo Alle
- (C) CITY: Bagsvaerd
- (E) COUNTRY: DENMARK
- (F) ZIP: DK-2880
- (v) COMPUTER READABLE FORM:
- 20 (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- 25 (vi) CURRENT APPLICATION DATA:
- (A) APPLICATION NUMBER:
- (B) FILING DATE:
- (C) CLASSIFICATION:
- 30 (viii) ATTORNEY/AGENT INFORMATION:
- (A) NAME: Thalsoe-Madsen, Birgit
- (ix) TELECOMMUNICATION INFORMATION:
- 35 (A) TELEPHONE: +45 4444 8888
- (B) TELEFAX: +45 4449 3256
- (C) TELEX: 37304

(2) INFORMATION FOR SEQ ID NO:1:

- 40 (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 1060 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- 45 (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA
- (iii) HYPOTHETICAL: NO
- 50 (vi) ORIGINAL SOURCE:
- (A) ORGANISM: Humicola insolens
- (B) STRAIN: DSM 1800

(ix) FEATURE:

(A) NAME/KEY: mat_peptide

(B) LOCATION: 73..927

5 (ix) FEATURE:

(A) NAME/KEY: sig_peptide

(B) LOCATION: 10..72

10 (ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 10..927

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

15 GGATCCAAG ATG CGT TCC TCC CCC CTC CTC CCG TCC GCC GGT GTG GCC 48
Met Arg Ser Ser Pro Leu Leu Pro Ser Ala Val Val Ala
-21 -20 -15 -10

20 GCC CTG CCG GTG TTG GCC CTT GCC GCT GAT GGC AGG TCC ACC CGC TAC 96
Ala Leu Pro Val Leu Ala Leu Ala Ala Asp Gly Arg Ser Thr Arg Tyr
-5 1 5

TGG GAC TGC TGC AAG CCT TCG TGC GGC TGG GCC AAG AAG GCT CCC GTG 144
25 Trp Asp Cys Cys Lys Pro Ser Cys Gly Trp Ala Lys Lys Ala Pro Val
10 15 20

AAC CAG CCT GTC TTT TCC TGC AAC GCC AAC TTC CAG CGT ATC ACG GAC 192
30 25 Asn Gln Pro Val Phe Ser Cys Asn Ala Asn Phe Gln Arg Ile Thr Asp
30 35 40

TTC GAC GCC AAG TCC GGC TGC GAG CCG GGC GGT GTC GCC TAC TCG TGC 240
Phe Asp Ala Lys Ser Gly Cys Glu Pro Gly Gly Val Ala Tyr Ser Cys
45 50 55

35 GCC GAC CAG ACC CCA TGG GCT GTG AAC GAC GAC TTC GCG CTC GGT TTT 288
Ala Asp Gln Thr Pro Trp Ala Val Asn Asp Asp Phe Ala Leu Gly Phe
60 65 70

40 GCT GCC ACC TCT ATT GCC GGC AGC AAT GAG GCG GGC TGG TGC TGC GCC 336
Ala Ala Thr Ser Ile Ala Gly Ser Asn Glu Ala Gly Trp Cys Cys Ala
75 80 85

TGC TAC GAG CTC ACC TTC ACA TCC GGT CCT GTT GCT GGC AAG AAG ATG 384
45 Cys Tyr Glu Leu Thr Phe Thr Ser Gly Pro Val Ala Gly Lys Lys Met
90 95 100

GTC GTC CAG TCC ACC AGC ACT GGC GGT GAT CTT GGC AGC AAC CAC TTC 432
50 105 Val Val Gln Ser Thr Ser Thr Gly Gly Asp Leu Gly Ser Asn His Phe
110 115 120

GAT CTC AAC ATC CCC GGC GGC GGC GTC GGC ATC TTC GAC GGA TGC ACT 480
Asp Leu Asn Ile Pro Gly Gly Gly Val Gly Ile Phe Asp Gly Cys Thr
125 130 135

| | | |
|----|---|------|
| | OCC CAG TTC GGC GGT CTG OCC GGC CAG OGC TAC GGC GGC ATC TCG TCC | 528 |
| | Pro Gln Phe Gly Gly Leu Pro Gly Gln Arg Tyr Gly Gly Ile Ser Ser | |
| | 140 145 150 | |
| 5 | OGC AAC GAG TGC GAT OGG TTC OCC GAC GOC CTC AAG OCC GGC TGC TAC | 576 |
| | Arg Asn Glu Cys Asp Arg Phe Pro Asp Ala Leu Lys Pro Gly Cys Tyr | |
| | 155 160 165 | |
| | TGG OGC TTC GAC TGG TTC AAG AAC GOC GAC AAT OCG AGC TTC AGC TTC | 624 |
| 10 | Trp Arg Phe Asp Trp Phe Lys Asn Ala Asp Asn Pro Ser Phe Ser Phe | |
| | 170 175 180 | |
| | OGT CAG GTC CAG TGC CCA GOC GAG CTC GTC GCT OGC ACC GGA TGC OGC | 672 |
| | Arg Gln Val Gln Cys Pro Ala Glu Leu Val Ala Arg Thr Gly Cys Arg | |
| 15 | 185 190 195 200 | |
| | OGC AAC GAC GAC GGC AAC TTC OCT GOC GTC CAG ATC CCC TCC AGC AGC | 720 |
| | Arg Asn Asp Asp Gly Asn Phe Pro Ala Val Gln Ile Pro Ser Ser Ser | |
| | 205 210 215 | |
| 20 | ACC AGC TCT CCG GTC AAC CAG OCT ACC AGC ACC AGC ACC ACG TCC ACC | 768 |
| | Thr Ser Ser Pro Val Asn Gln Pro Thr Ser Thr Ser Thr Thr Ser Thr | |
| | 220 225 230 | |
| 25 | TCC ACC ACC TCG AGC CCG CCA GTC CAG OCT ACG ACT CCC AGC GGC TGC | 816 |
| | Ser Thr Thr Ser Ser Pro Pro Val Gln Pro Thr Thr Pro Ser Gly Cys | |
| | 235 240 245 | |
| | ACT GCT GAG AGG TGG GCT CAG TGC GGC GGC AAT GGC TGG AGC GGC TGC | 864 |
| 30 | Thr Ala Glu Arg Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys | |
| | 250 255 260 | |
| | ACC ACC TGC GTC GCT GGC AGC ACT TGC ACG AAG ATT AAT GAC TGG TAC | 912 |
| | Thr Thr Cys Val Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr | |
| 35 | 265 270 275 280 | |
| | CAT CAG TGC CTG TAGACG CAGG GCAGCTTGAG GGCTTTACTG GTGGGCGCAA | 964 |
| | His Gln Cys Leu | |
| | 285 | |
| 40 | CGAAATGACA CTCCCAATCA CTGTATTAGT TCTTGTACAT AATTTCGTCA TCCCTCCAGG | 1024 |
| | GATTGTGACA TAAATGCAAT GAGGAACAAT GAGTAC | 1060 |
| 45 | | |

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 305 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10 (*i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Met Arg Ser Ser Pro Leu Leu Pro Ser Ala Val Val Ala Ala Leu Pro
-21 -20                -15                -10

15 Val Leu Ala Leu Ala Ala Asp Gly Arg Ser Thr Arg Tyr Trp Asp Cys
   -5                1                5                10

Cys Lys Pro Ser Cys Gly Trp Ala Lys Lys Ala Pro Val Asn Gln Pro
                15                20                25

20 Val Phe Ser Cys Asn Ala Asn Phe Gln Arg Ile Thr Asp Phe Asp Ala
   30                35                40

Lys Ser Gly Cys Glu Pro Gly Gly Val Ala Tyr Ser Cys Ala Asp Gln
25   45                50                55

Thr Pro Trp Ala Val Asn Asp Asp Phe Ala Leu Gly Phe Ala Ala Thr
   60                65                70                75

30 Ser Ile Ala Gly Ser Asn Glu Ala Gly Trp Cys Cys Ala Cys Tyr Glu
                80                85                90

Leu Thr Phe Thr Ser Gly Pro Val Ala Gly Lys Lys Met Val Val Gln
                95                100               105

35 Ser Thr Ser Thr Gly Gly Asp Leu Gly Ser Asn His Phe Asp Leu Asn
   110               115               120

Ile Pro Gly Gly Gly Val Gly Ile Phe Asp Gly Cys Thr Pro Gln Phe
40   125               130               135

Gly Gly Leu Pro Gly Gln Arg Tyr Gly Gly Ile Ser Ser Arg Asn Glu
140               145               150               155

45 Cys Asp Arg Phe Pro Asp Ala Leu Lys Pro Gly Cys Tyr Trp Arg Phe
                160                165                170

Asp Trp Phe Lys Asn Ala Asp Asn Pro Ser Phe Ser Phe Arg Gln Val
                175                180                185

50 Gln Cys Pro Ala Glu Leu Val Ala Arg Thr Gly Cys Arg Arg Asn Asp
   190                195                200

Asp Gly Asn Phe Pro Ala Val Gln Ile Pro Ser Ser Ser Thr Ser Ser
55   205                210                215

```

Pro Val Asn Gln Pro Thr Ser Thr Ser Thr Thr Ser Thr Ser Thr Thr
220 225 230 235

5 Ser Ser Pro Pro Val Gln Pro Thr Thr Pro Ser Gly Cys Thr Ala Glu
240 245 250

Arg Trp Ala Gln Cys Gly Gly Asn Gly Trp Ser Gly Cys Thr Thr Cys
255 260 265

10 Val Ala Gly Ser Thr Cys Thr Lys Ile Asn Asp Trp Tyr His Gln Cys
270 275 280

Leu
15

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1473 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

10

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

15

(vi) ORIGINAL SOURCE:

- (A) ORGANISM: *Fusarium oxysporum*
 (B) STRAIN: DSM 2672

(ix) FEATURE:

20

- (A) NAME/KEY: CDS
 (B) LOCATION: 97..1224

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

25

GAATTGOGGG CCGCTCATTC ACTTCATTCA TTCTTTAGAA TTACATACAC TCTCTTTCAA 60

AACAGTCACT CTTTAAACAA AACAACTTTT GCAACA ATG CGA TCT TAC ACT CTT 114

Met Arg Ser Tyr Thr Leu
 1 5

30

CTC GGC CTG GGC GGC CCT CTC GGC GTG AGT GCT GCT TCT GGA AGC GGT 162

Leu Ala Leu Ala Gly Pro Leu Ala Val Ser Ala Ala Ser Gly Ser Gly
 10 15 20

35

CAC TCT ACT CGA TAC TGG GAT TGC TGC AAG CCT TCT TGC TCT TGG AGC 210

His Ser Thr Arg Tyr Trp Asp Cys Cys Lys Pro Ser Cys Ser Trp Ser
 25 30 35

40

GGA AAG GCT GCT GTC AAC GCC CCT GCT TTA ACT TGT GAT AAG AAC GAC 258

Gly Lys Ala Ala Val Asn Ala Pro Ala Leu Thr Cys Asp Lys Asn Asp
 40 45 50

45

AAC CCC ATT TCC AAC ACC AAT GCT GTC AAC GGT TGT GAG GGT GGT GGT 306

Asn Pro Ile Ser Asn Thr Asn Ala Val Asn Gly Cys Glu Gly Gly Gly
 55 60 65 70

50

TCT GCT TAT GCT TGC ACC AAC TAC TCT CCC TGG GCT GTC AAC GAT GAG 354

Ser Ala Tyr Ala Cys Thr Asn Tyr Ser Pro Trp Ala Val Asn Asp Glu
 75 80 85

55

CTT GGC TAC GGT TTC GCT GCT ACC AAG ATC TCC GGT GGC TCC GAG GCC 402

Leu Ala Tyr Gly Phe Ala Ala Thr Lys Ile Ser Gly Gly Ser Glu Ala
 90 95 100

| | | |
|----|--|------|
| | AGC TGG TGC TGT GCT TGC TAT GCT TTG ACC TTC ACC ACT GGC CCC GTC Ser Trp Cys Cys Ala Cys Tyr Ala Leu Thr Phe Thr Thr Gly Pro Val | 450 |
| | 105 110 115 | |
| 5 | AAG GGC AAG AAG ATG ATC GTC CAG TCC ACC AAC ACT GGA GGT GAT CTC Lys Gly Lys Lys Met Ile Val Gln Ser Thr Asn Thr Gly Gly Asp Leu | 498 |
| | 120 125 130 | |
| 10 | GGC GAC AAC CAC TTC GAT CTC ATG ATG CCC GGC GGT GGT GTC GGT ATC Gly Asp Asn His Phe Asp Leu Met Met Pro Gly Gly Gly Val Gly Ile | 546 |
| | 135 140 145 150 | |
| 15 | TTC GAC GGC TGC ACC TCT GAG TTC GGC AAG GCT CTC GGC GGT GGC CAG Phe Asp Gly Cys Thr Ser Glu Phe Gly Lys Ala Leu Gly Gly Ala Gln | 594 |
| | 155 160 165 | |
| 20 | TAC GGC GGT ATC TCC TCC CGA AGC GAA TGT GAT AGC TAC CCC GAG CTT Tyr Gly Gly Ile Ser Ser Arg Ser Glu Cys Asp Ser Tyr Pro Glu Leu | 642 |
| | 170 175 180 | |
| 25 | CTC AAG GAC GGT TGC CAC TGG CGA TTC GAC TGG TTC GAG AAC GCC GAC Leu Lys Asp Gly Cys His Trp Arg Phe Asp Trp Phe Glu Asn Ala Asp | 690 |
| | 185 190 195 | |
| 30 | AAC CCT GAC TTC ACC TTT GAG CAG GTT CAG TGC CCC AAG GCT CTC CTC Asn Pro Asp Phe Thr Phe Glu Gln Val Gln Cys Pro Lys Ala Leu Leu | 738 |
| | 200 205 210 | |
| 35 | GAC ATC AGT GGA TGC AAG CGT GAT GAC GAC TCC AGC TTC OCT GCC TTC Asp Ile Ser Gly Cys Lys Arg Asp Asp Asp Ser Ser Phe Pro Ala Phe | 786 |
| | 215 220 225 230 | |
| 40 | AAG GTT GAT ACC TCG GCC AGC AAG CCC CAG CCC TCC AGC TCC GCT AAG Lys Val Asp Thr Ser Ala Ser Lys Pro Gln Pro Ser Ser Ser Ala Lys | 834 |
| | 235 240 245 | |
| 45 | AAG ACC ACC TCC GCT GCT GCT GCC GCT CAG CCC CAG AAG ACC AAG GAT Lys Thr Thr Ser Ala Ala Ala Ala Gln Pro Gln Lys Thr Lys Asp | 882 |
| | 250 255 260 | |
| 50 | TCC GCT CCT GTT GTC CAG AAG TCC TCC ACC AAG OCT GCC GCT CAG CCC Ser Ala Pro Val Val Gln Lys Ser Ser Thr Lys Pro Ala Ala Gln Pro | 930 |
| | 265 270 275 | |
| 55 | GAG OCT ACT AAG CCC GCC GAC AAG CCC CAG ACC GAC AAG OCT GTC GCC Glu Pro Thr Lys Pro Ala Asp Lys Pro Gln Thr Asp Lys Pro Val Ala | 978 |
| | 280 285 290 | |
| 60 | ACC AAG OCT GCT GCT ACC AAG CCC GTC CAA OCT GTC AAC AAG CCC AAG Thr Lys Pro Ala Ala Thr Lys Pro Val Gln Pro Val Asn Lys Pro Lys | 1026 |
| | 295 300 305 310 | |
| 65 | ACA ACC CAG AAG GTC CGT GGA ACC AAA ACC CGA GGA AGC TGC CCG GCC Thr Thr Gln Lys Val Arg Gly Thr Lys Thr Arg Gly Ser Cys Pro Ala | 1074 |
| | 315 320 325 | |

| | | |
|----|--|------|
| | AAG ACT GAC GCT AOC GOC AAG GOC TOC GTT GTC OCT GCT TAT TAC CAG | 1122 |
| | Lys Thr Asp Ala Thr Ala Lys Ala Ser Val Val Pro Ala Tyr Tyr Gln | |
| | 330 335 340 | |
| 5 | TGT GGT GGT TCC AAG TOC GCT TAT CCC AAC GGC AAC CTC GCT TGC GCT | 1170 |
| | Cys Gly Gly Ser Lys Ser Ala Tyr Pro Asn Gly Asn Leu Ala Cys Ala | |
| | 345 350 355 | |
| | ACT GGA AGC AAG TGT GTC AAG CAG AAC GAG TAC TAC TOC CAG TGT GTC | 1218 |
| 10 | Thr Gly Ser Lys Cys Val Lys Gln Asn Glu Tyr Tyr Ser Gln Cys Val | |
| | 360 365 370 | |
| | CCC AAC TAAATGGTAG ATOCATOGGT TGTGGAAGAG ACTATGGGTC TCAGAAGGGA | 1274 |
| | Pro Asn | |
| 15 | 375 | |
| | TCCTCICATG AGCAGGCTTG TCATTGTATA GCATGGCATC CIGGAACCAAG TGTTOGACCC | 1334 |
| | TGTGTGTACA TAGTATATCT TCATTGTATA TATTTAGACA CATAGATAGC CTCGTGTACG | 1394 |
| 20 | OGACAACTGG CTACAAAAGA CTTGGCAGGC TTGTTCAATA TTGACACAGT TTCTTCATA | 1454 |
| | AAAAAAAAAA AAAAAAAAAA | 1473 |

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 376 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

10

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Arg Ser Tyr Thr Leu Leu Ala Leu Ala Gly Pro Leu Ala Val Ser
1 5 10 15

Ala Ala Ser Gly Ser Gly His Ser Thr Arg Tyr Trp Asp Cys Cys Lys
20 25 30

Pro Ser Cys Ser Trp Ser Gly Lys Ala Ala Val Asn Ala Pro Ala Leu
20 35 40 45

Thr Cys Asp Lys Asn Asp Asn Pro Ile Ser Asn Thr Asn Ala Val Asn
50 55 60

25 Gly Cys Glu Gly Gly Gly Ser Ala Tyr Ala Cys Thr Asn Tyr Ser Pro
65 70 75 80

Trp Ala Val Asn Asp Glu Leu Ala Tyr Gly Phe Ala Ala Thr Lys Ile
85 90 95

30 Ser Gly Gly Ser Glu Ala Ser Trp Cys Cys Ala Cys Tyr Ala Leu Thr
100 105 110

Phe Thr Thr Gly Pro Val Lys Gly Lys Lys Met Ile Val Gln Ser Thr
35 115 120 125

Asn Thr Gly Gly Asp Leu Gly Asp Asn His Phe Asp Leu Met Met Pro
130 135 140

40 Gly Gly Gly Val Gly Ile Phe Asp Gly Cys Thr Ser Glu Phe Gly Lys
145 150 155 160

Ala Leu Gly Gly Ala Gln Tyr Gly Gly Ile Ser Ser Arg Ser Glu Cys
165 170 175

45 Asp Ser Tyr Pro Glu Leu Leu Lys Asp Gly Cys His Trp Arg Phe Asp
180 185 190

Trp Phe Glu Asn Ala Asp Asn Pro Asp Phe Thr Phe Glu Gln Val Gln
50 195 200 205

Cys Pro Lys Ala Leu Leu Asp Ile Ser Gly Cys Lys Arg Asp Asp Asp
210 215 220

55 Ser Ser Phe Pro Ala Phe Lys Val Asp Thr Ser Ala Ser Lys Pro Gln
225 230 235 240

CLAIMS

1. A cellulase preparation consisting essentially of a homogenous endoglucanase component which is immunoreactive with an antibody raised against a highly purified ~43 kD endoglucanase derived from Humicola insolens, DSM 1800, or which is homologous to said ~43 kD endoglucanase.

2. A cellulase preparation according to claim 1, wherein the endoglucanase component has an endoglucanase activity of at least 50 CMC-endoase units/mg of protein.

10 3. A cellulase preparation according to claim 2, wherein the endoglucanase component has an endoglucanase activity of at least 60 CMC-endoase units/mg of total protein, in particular at least 90 CMC-endoase units/mg of total protein, and preferably at least 100 CMC-endoase units/mg of total protein.

15 4. A cellulase preparation according to claim 1, wherein the endoglucanase component has essentially no cello-biohydrolase activity.

5. A cellulase preparation according to any of claims 1-4, wherein the endoglucanase component has an isoelectric point 20 of about 5.1.

6. An enzyme exhibiting endoglucanase activity, which enzyme has the amino acid sequence shown in the appended Sequence Listing ID#2, or a homologue thereof exhibiting endoglucanase activity.

25 7. An endoglucanase enzyme according to claim 6 which is producible by a species of Humicola, e.g. Humicola insolens.

8. An enzyme exhibiting endoglucanase activity, which enzyme has the amino acid sequence shown in the appended Sequence Listing ID#4, or a homologue thereof exhibiting 30 endoglucanase activity.

9. An endoglucanase enzyme according to claim 8 which is producible by a species of Fusarium, e.g. Fusarium oxysporum.

10. A DNA construct comprising a DNA sequence encoding an endoglucanase enzyme as claimed in any of claims 6-9.

11. A DNA construct according to claim 10, wherein the DNA sequence is as shown in the appended Sequence Listings ID#1 or ID#3 or a modification thereof.

12. An expression vector which carries an inserted DNA sequence according to claim 10 or 11.

13. A cell which is transformed with a DNA construct according to claim 10 or 11 or with an expression vector according to claim 12.

14. A cell according to claim 13 which is a fungal cell, e.g. belonging to a strain of Trichoderma or Aspergillus, in particular Aspergillus oryzae or Aspergillus niger, or a yeast cell, e.g. belonging to a strain of Hansenula or Saccharomyces, e.g. Saccharomyces cerevisiae.

15. A process for producing an endoglucanase enzyme as defined in any of claims 6-9, the process comprising culturing a cell according to claim 13 or 14 in a suitable culture medium under conditions permitting the expression of the endoglucanase enzyme, and recovering the endoglucanase enzyme from the culture.

16. A detergent additive containing a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme according to any of claims 6-9, preferably in the form of a non-dusting granulate, stabilized liquid or protected enzyme.

17. A detergent additive according to claim 16 which contains 1-500, preferably 5-250, most preferably 10-100, mg of enzyme protein per gram of the additive.

18. A detergent additive according to claim 16 which additionally comprises another enzyme such as a protease, lipase, peroxidase and/or amylase.

19. A detergent additive according to claim 18, wherein the protease is one which has a higher degree of specificity than Bacillus lentus serine protease.

20. A detergent additive according to claim 19, wherein the protease is subtilisin Novo or a variant thereof, a protease derivable from Nocardia dassonvillei NRRL 18133, a serine protease specific for glutamic and aspartic acid,

producible by Bacillus licheniformis, or a trypsin-like protease producible by Fusarium sp. DSM 2672.

21. A detergent composition comprising a cellulase preparation according to any of claims 1-5 or an endoglucanase 5 enzyme according to any of claims 6-9.

22. A detergent composition according to claim 21, which additionally comprises another enzyme such as a protease, lipase, peroxidase and/or amylase.

23. A detergent composition according to claim 22, 10 wherein the protease is one which has a higher degree of specificity than Bacillus lentus serine protease.

24. A detergent composition according to claim 23, wherein the protease is subtilisin Novo or a variant thereof, a protease derivable from Nocardia dassonvillei NRRL 18133, a 15 serine protease specific for glutamic and aspartic acid, producible by Bacillus licheniformis, or a trypsin-like protease producible by Fusarium sp. DSM 2672.

25. A detergent composition according to claim 21, wherein the cellulase preparation or endoglucanase enzyme is 20 present in a concentration corresponding to 0.01-100, preferably 0.05-60, and most preferably 0.1-20, mg of enzyme protein per liter washing solution.

26. A detergent composition comprising a detergent additive according to any of claims 16-20.

25 27. A method of reducing the rate at which cellulose-containing fabrics become harsh or of reducing the harshness of cellulose-containing fabrics, the method comprising treating cellulose-containing fabrics with a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme 30 according to any of claims 6-9.

28. A method of providing colour clarification of coloured cellulose-containing fabrics, the method comprising treating coloured cotton-containing fabrics with a cellulase preparation according to any of claims 1-5 or an endoglucanase 35 enzyme according to any of claims 6-9.

29. A method of providing a localized variation in colour of coloured cellulose-containing fabrics, the method comprising treating coloured cotton-containing fabrics with a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme according to any of claims 6-9.

30. A method according to any of claims 27, 28 or 29, wherein the treatment of the fabrics with the cellulase preparation is carried out during soaking, washing or rinsing of the fabrics.

10 31. A method of improving the drainage properties of pulp, the method comprising treating paper pulp with a cellulase preparation according to any of claims 1-5 or an endoglucanase enzyme according to any of claims 6-9.

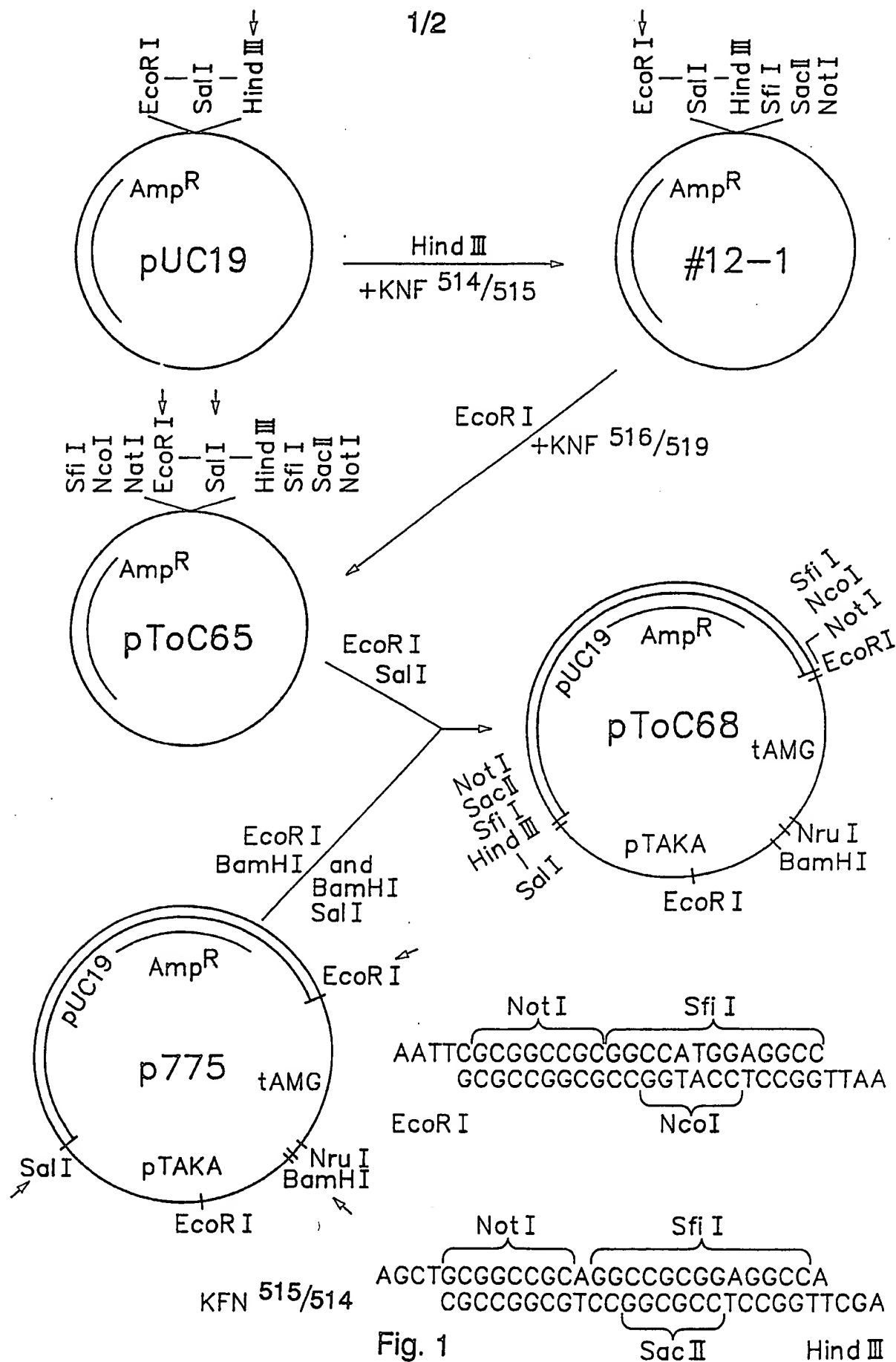


Fig. 1

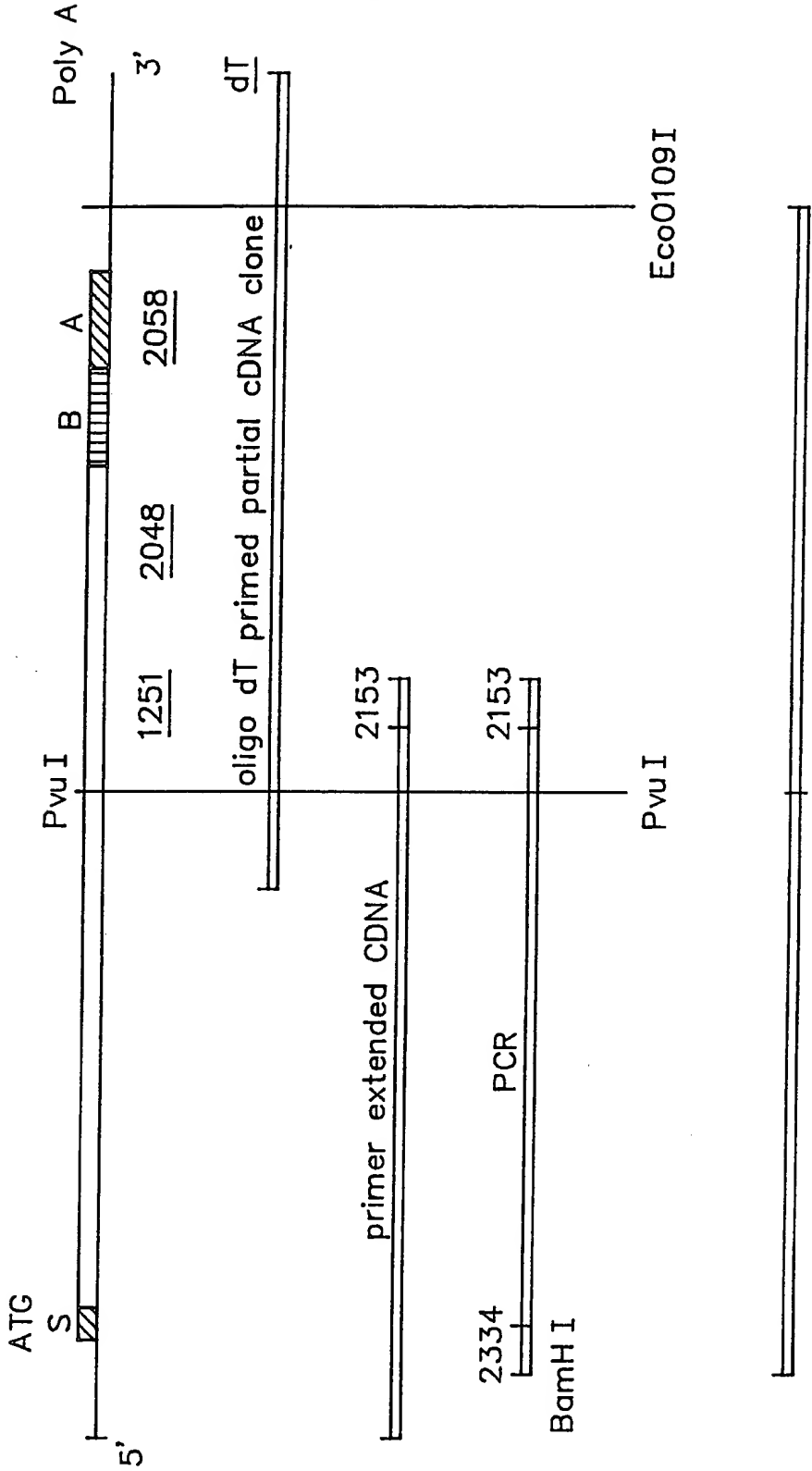


Fig. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/DK 91/00123

| I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ⁶ According to International Patent Classification (IPC) or to both National Classification and IPC IPC5: C 12 N 9/42, C 12 N 15/56, C 11 D 3/387 | | | | | | | | | | | | | | | | | | | | |
|--|---|-------------------------------------|--|--|--|--|--|-----------|---|----|------|---|---|-----------|---|---|-----------|--|----|--|
| II. FIELDS SEARCHED <div style="text-align: center; border: 1px solid black; padding: 2px;">Minimum Documentation Searched⁷</div> <table style="width: 100%; border-collapse: collapse;"> <tr> <th style="width: 20%; border: 1px solid black; padding: 2px;">Classification System</th> <th style="border: 1px solid black; padding: 2px;">Classification Symbols</th> </tr> <tr> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">IPC5</td> <td style="border: 1px solid black; padding: 5px; vertical-align: top;">C 11 D; C 12 N</td> </tr> </table> <div style="border: 1px solid black; padding: 2px; text-align: center; margin-top: 5px;">Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched⁸</div> | | | Classification System | Classification Symbols | IPC5 | C 11 D; C 12 N | | | | | | | | | | | | | | |
| Classification System | Classification Symbols | | | | | | | | | | | | | | | | | | | |
| IPC5 | C 11 D; C 12 N | | | | | | | | | | | | | | | | | | | |
| SE,DK,FI,NO classes as above | | | | | | | | | | | | | | | | | | | | |
| III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹ <table border="1" style="width: 100%; border-collapse: collapse;"> <thead> <tr> <th style="width: 10%; padding: 2px;">Category *</th> <th style="width: 60%; padding: 2px;">Citation of Document,¹¹ with indication, where appropriate, of the relevant passages¹²</th> <th style="width: 30%; padding: 2px;">Relevant to Claim No.¹³</th> </tr> </thead> <tbody> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">X</td> <td style="padding: 5px;">WO, A1, 8909259 (NOVO INDUSTRI A/S) 5 October 1989, see the whole document</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-5,16-31</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td style="text-align: center; vertical-align: top; padding: 5px;">6-15</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">US, A, 4435307 (BARBESGAARD ET AL) 6 March 1984, see the whole document</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-5,16-31</td> </tr> <tr> <td style="text-align: center; vertical-align: top; padding: 5px;">A</td> <td style="padding: 5px;">Chemical Abstracts, volume 107, no. 9, 31 August 1987, (Columbus, Ohio, US), Poulsen, Otto M et al: "Purification of an extracellular cellulose-binding endoglucanase of Cellulomonas sp. ATCC 21399 by affinity chromatography on phosphoric acid-swollen cellulose. ", see page 292, abstract 73161r, & Biotechnol.Bioeng. 1987, 29(7), 799- 804</td> <td style="text-align: center; vertical-align: top; padding: 5px;">1-5,16-31</td> </tr> <tr> <td></td> <td style="text-align: center; vertical-align: top; padding: 5px;">--</td> <td></td> </tr> </tbody> </table> | | | Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | X | WO, A1, 8909259 (NOVO INDUSTRI A/S) 5 October 1989, see the whole document | 1-5,16-31 | A | -- | 6-15 | A | US, A, 4435307 (BARBESGAARD ET AL) 6 March 1984, see the whole document | 1-5,16-31 | A | Chemical Abstracts, volume 107, no. 9, 31 August 1987, (Columbus, Ohio, US), Poulsen, Otto M et al: "Purification of an extracellular cellulose-binding endoglucanase of Cellulomonas sp. ATCC 21399 by affinity chromatography on phosphoric acid-swollen cellulose. ", see page 292, abstract 73161r, & Biotechnol.Bioeng. 1987, 29(7), 799- 804 | 1-5,16-31 | | -- | |
| Category * | Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ | | | | | | | | | | | | | | | | | | |
| X | WO, A1, 8909259 (NOVO INDUSTRI A/S) 5 October 1989, see the whole document | 1-5,16-31 | | | | | | | | | | | | | | | | | | |
| A | -- | 6-15 | | | | | | | | | | | | | | | | | | |
| A | US, A, 4435307 (BARBESGAARD ET AL) 6 March 1984, see the whole document | 1-5,16-31 | | | | | | | | | | | | | | | | | | |
| A | Chemical Abstracts, volume 107, no. 9, 31 August 1987, (Columbus, Ohio, US), Poulsen, Otto M et al: "Purification of an extracellular cellulose-binding endoglucanase of Cellulomonas sp. ATCC 21399 by affinity chromatography on phosphoric acid-swollen cellulose. ", see page 292, abstract 73161r, & Biotechnol.Bioeng. 1987, 29(7), 799- 804 | 1-5,16-31 | | | | | | | | | | | | | | | | | | |
| | -- | | | | | | | | | | | | | | | | | | | |
| <div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p>* Special categories of cited documents: ¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div> | | | | | | | | | | | | | | | | | | | | |
| IV. CERTIFICATION <table style="width: 100%; border-collapse: collapse;"> <tr> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of the Actual Completion of the International Search 20th August 1991 </td> <td style="width: 50%; border: 1px solid black; padding: 5px;"> Date of Mailing of this International Search Report 1991 -10- 21 </td> </tr> <tr> <td style="border: 1px solid black; padding: 5px;"> International Searching Authority <div style="text-align: center; margin-top: 10px;">SWEDISH PATENT OFFICE</div> </td> <td style="border: 1px solid black; padding: 5px;"> Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> Yvonne Siösteen </div> </td> </tr> </table> | | | Date of the Actual Completion of the International Search 20th August 1991 | Date of Mailing of this International Search Report 1991 -10- 21 | International Searching Authority <div style="text-align: center; margin-top: 10px;">SWEDISH PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> Yvonne Siösteen </div> | | | | | | | | | | | | | | |
| Date of the Actual Completion of the International Search 20th August 1991 | Date of Mailing of this International Search Report 1991 -10- 21 | | | | | | | | | | | | | | | | | | | |
| International Searching Authority <div style="text-align: center; margin-top: 10px;">SWEDISH PATENT OFFICE</div> | Signature of Authorized Officer <div style="text-align: center; margin-top: 10px;"> Yvonne Siösteen </div> | | | | | | | | | | | | | | | | | | | |

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|--|----------------------|
| Category * | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claim No |
| A | Chemical Abstracts, volume 106, no. 17, 27 April 1987, (Columbus, Ohio, US), Klesov, A.A. et al.: "Thermostable 1,4-beta-endoglucanase from Myceliophthora thermophila: purification and characterization. ", see page 302, abstract 134159z, & Prikl.Biokhim.Mikrobiol. 1987, 23(1), 44- 50 -- | 1-5,16-31 |
| A | Chemical Abstracts, volume 109, no. 23, 5 December 1988, (Columbus, Ohio, US), Hayashida, Shinsaku et al.: "Cellulases of Humicola insolens and Humicola grisea ", see page 295, abstract 207112c, & Methods Enzymol.(Biomass, Pt. A) 1988, 160(), 323- 332 -- | 6-7,10-15 |
| A | Chemical Abstracts, volume 114, no. 15, 15 April 1991, (Columbus, Ohio, US), Ortega Jacobo: "Production of extracellular cellulolytic enzymes by Fusarium oxysporum f.sp. lycopersici ", see page 619, abstract 141530g, & Tex. J. Sci. 1990, 42(4), 405- 409 -- | 9-15 |
| A | Chemical Abstracts, volume 105, no. 13, 29 September 1986, (Columbus, Ohio, US), Rao, Mala et al.: "Purification, characterization, and synergistic action of endoglucanases from Fusarium lini ", see page 307, abstract 110894p, & Biotechnol. Bioeng. 1986, 28(7), 1100-1105 -- | 8-15 |
| A | Chemical Abstracts, volume 105, no. 3, 21 July 1986, (Columbus, Ohio, US), Hayashida, Shinsaku et al.: "Production and characteristics of Avicel-disintegrating endoglucanase from a protease-negative Humicola grisea var. thermoidea mutant. ", see page 316, abstract 20820g, & Appl. Environ. Microbiol. 1986, 51(5), 1041-1046 -- ----- | 6-7,10-15 |

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers....., because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claim numbers....., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:

See attached sheet

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the the claims. It is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

The inventions claimed are composed of the following three different inventions:

- 1) The indefinite claims 1-5 relate to a cellulase preparation consisting essentially of a homogenous endoglucanase component.
- 2) Claims 6-7 and partly claims 10-31 relate to a specific enzyme exhibiting endoglucanase activity. The enzyme is being defined by its amino acid sequence.
- 3) Claims 8-9 and partly claims 10-31 relate to a specific enzyme exhibiting endoglucanase activity. The enzyme is being defined by its amino acid sequence.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.PCT/DK 91/00123**

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the Swedish Patent Office EDP file on **91-08-30**
The Swedish Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---------------------|----------------------------|---------------------|
| WO-A1- 8909259 | 89-10-05 | EP-A- 0406314 | 91-01-09 |
| US-A- 4435307 | 84-03-06 | AT-B- 384442 | 87-11-10 |
| | | BE-A- 888632 | 81-10-29 |
| | | CH-A- 663511 | 87-12-31 |
| | | DE-A- 3117250 | 82-04-01 |
| | | FR-A-B- 2481712 | 81-11-06 |
| | | GB-A-B- 2075028 | 81-11-11 |
| | | JP-C- 1443072 | 88-06-08 |
| | | JP-A- 57023699 | 82-02-06 |
| | | JP-B- 61016316 | 86-04-30 |
| | | NL-A- 8102123 | 81-11-16 |